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DIRECT DETECTION OF FREE RADICAL GENERATION IN AN *IN VIVO* MODEL OF ACUTE LUNG INJURY

PAUL G. MURPHY, DAVID S. MYERS, NIGEL R. WEBSTER and J. GARETH JONES

Department of Anaesthesia, University of Leeds, 24 Hyde Terrace, Leeds LS2 9LN, U.K.

MICHAEL J. DAVIES‡

Department of Chemistry, University of York, Heslington, York YO1 5DD, U.K.

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Electron spin resonance (ESR) spectroscopy has been used to provide direct evidence that free radical production occurs in an *in vivo* model of acute lung injury. Two experimental groups of rabbits were given the spin trap α-phenyl N-*tert*.-butyl nitrone (PBN), together with endotoxin in the test group, and saline in the control group. Both groups were subsequently briefly ventilated with air containing cigarette smoke. Plasma samples from the endotoxin pretreated group showed a sudden burst of radical formation, detected as PBN spin adduct, which peaked in the first ten minutes after smoke exposure. No signals were detected in the control group. Permeability of the alveolar capillary barrier of the lung, measured by the clearance of ^{99m}Tc-DTPA, demonstrated significantly greater damage following smoke in the endotoxin primed animals than in the controls. Temporal studies suggest that this increase in permeability occurred after a burst of radical production. These studies provide supportive evidence for the hypothesis that endotoxin promotes the accumulation of a population of primed white cells within the lung, which when triggered by cigarette smoke, are able to generate a burst of free radicals which produce tissue damage and acute lung injury.

KEY WORDS: Endotoxin, smoke, radicals, spin trap, lung injury, ESR.

INTRODUCTION

Acute lung injury is characterised by damage to the pulmonary structures across which gas exchange takes place, namely the alveolar-capillary barrier (ACB).[1] The most severe form of this condition, the Adult Respiratory Distress Syndrome (ARDS), requires artificial ventilation with high inspired oxygen fractions and produces a mortality rate of greater than 50%.[2] Sepsis is a major cause of acute lung injury,[3] and it hypothesised that the ACB may be particularly vulnerable to damage mediated by the subsequent inflammatory/immune response.[2]

A myriad of cellular and humoral mediators of such tissue injury have been proposed, included in which are oxygen derived free radicals (ODFRs).

There are several reasons why ODFRs might be generated during acute lung injury.[4] There is abundant evidence that neutrophils and other white cells play an

‡To whom correspondence should be addressed.

important role in the initial pulmonary damage in patients who develop acute lung injury,[5] and that these activated white cells are able to generate ODFRs. Artificial ventilation with high inspired fractions of oxygen, and reperfusion of ischaemic pulmonary tissue[6] are other potential mechanisms by which radicals might be generated within lung tissue in the critically ill patient.

The extent of damage to the ACB can be assessed experimentally by measuring the solute permeability of this barrier.[7] Whilst the integrity of the barrier normally keeps the alveolus dry, acute lung injury is associated with an increase in the permeability of the ACB and progressive fluid accumulation within the pulmonary interstitium and alveolae. Experimental work suggests that the barrier is vulnerable to oxidant mediated damage,[8] although a recent major review has highlighted the lack of direct evidence that radicals are generated during the evolution of such injury.[9]

Electron spin resonance (ESR) spectroscopy is a valuable technique for detecting and identifying radical production both *in vitro* and *in vivo*.[10,11] We have therefore applied this technique, using the spin trapping agent α-phenyl N-*tert*.-butyl nitrone (PBN), to examine radical generation in an *in vivo* model of acute lung injury. In this model, acute lung injury is induced with a combination of bacterial endotoxin and smoke inhalation, and assessed according to changes in the permeability of the alveolar epithelium to an aerosol of ^{99m}Tc-DTPA. We have previously shown that endotoxin alone has no effect on the integrity of the alveolar epithelium, but significantly amplifies smoke induced increases in lung permeability.[12,13]

MATERIALS AND METHODS

This study was carried out under a Home Office Project Licence, Licence No PPL 50/00467.

Animal Preparation

Male New Zealand White rabbits (3 to 3.5 kg) were sedated with 0.6 ml Hypnorm (fentanyl 0.315 mg/ml, fluanisole 10 mg/ml, Janssen) and an intravenous and intra-arterial cannula sited in one ear. Anaesthesia was induced intravenously with 0.1 ml aliquots of Hypnorm. A tracheostomy was performed under aseptic conditions and the animals ventilated with oxygen enriched air to normocapnia. The animals were kept on a heated operating table and the core temperature continuously monitored. Anaesthesia was maintained with an intravenous infusion of Hypnorm ($0.1\,ml\,kg^{-1}\,hr^{-1}$). At the end of the experiments the animals were sacrificed with an overdose of pentobarbitone.

Experimental Design

Two experimental groups of animals were defined (Figure 1; $n = 6$ in both cases). The test (endotoxin/smoke) group received $3\,\mu g\,kg^{-1}$ bacterial endotoxin (*E. coli* serotype 026 : B6, Sigma) dissolved in 10 ml sterile 0.9% NaCl, which was substituted by saline alone in the control (saline/smoke) group. One hour after endotoxin the animals were given $300\,mg\,kg^{-1}$ PBN (Aldrich) dissolved in 40 ml 0.9% sterile NaCl, which was infused over 45 min. Two hours after endotoxin the animals were briefly ventilated with cigarette smoke. This was delivered in 20 20 ml breaths of smoke

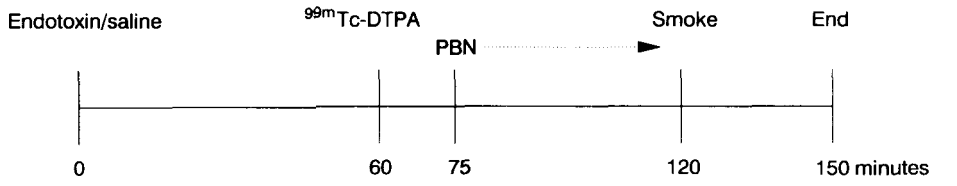
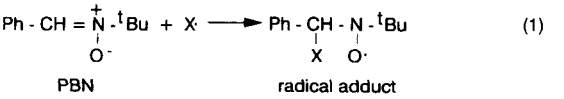


FIGURE 1 Experimental design.

drawn from a lit cigarette (University of Kentucky Research Cigarettes, 1R1) over 5 s directly into a syringe, which was then connected to the tracheostomy tube and the lungs inflated over a 4 s period. In order to prevent hypoxaemia during smoke exposure, each breath was interspersed with 5–6 ventilator breaths of oxygen enriched air.

It is essential to include a spin trap because of the short half lives of most radicals generated in *in vivo* systems, which would preclude direct detection. PBN was chosen as it is known to form relatively long-lived radical adducts with certain types of radical[14] ($X^{\cdot}$; reaction 1) and has been shown to be compatible



(1)

with normal physiological functions at low concentrations.[10,15,16] Experiments have shown that PBN can enter hepatocytes, brain, spleen and heart cells, and that spin adducts of PBN can cross cell membranes;[10,15–17] it has therefore been assumed that both PBN and its spin adducts can readily equilibrate between any cells involved and plasma.

ESR Analysis

Blood samples for ESR analysis were taken from the indwelling arterial cannula 5 min. before smoke (in triplicate), and 1, 2, 5, 10, 15 and 30 min. after smoke administration. Samples were taken using ice-cold heparinised syringes and immediately decanted into ice-cold plastic tubes. The plasma was separated within 5 min. of sampling by centrifugation at 3000 rpm for 10 min. at −4°C, and the plasma specimens then stored in liquid nitrogen until ESR analysis. Analysis of the samples for spin adducts was carried out within 2 weeks of the experiment on unmodified thawed plasma at room temperature using a standard aqueous cell and a Bruker ESP 300 ESR spectrometer equipped with 100 kHz modulation and a Bruker ER035M gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the field scan. Relative radical concentrations were measured as peak to peak line heights (in cm) on spectra recorded with identical spectrometer settings. Background spectrometer noise has not been subtracted from these measurements.

Measurement of Lung Permeability

The technique for assessing the permeability of the alveolar epithelium by measuring the clearance from the lungs of ^{99m}Tc-DTPA deposited upon the alveolar surface has been described in detail elsewhere.[18,19] The aerosol of radiotracer was given one hour

after endotoxin, and the permeability of the lung measured before and after smoke administration. Permeability is expressed in terms of a clearance half-time, $t_{1/2}$, which is calculated from the equation

$$t_{1/2} = \frac{\ln 2}{k}$$

where k is the slope of the curve derived from a plot of log radioactivity against time and calculated using a commercially available computer graphics package by least-squares linear regression analysis. As previously described,[18] correction was made for the accumulation of radioactivity within the interstitial spaces of the lung. Count rates were also corrected for (i) physical background activity, and (ii) decay of the isotope (^{99m}Tc has a half life of approximately 6 h).

Statistical Analysis

ESR signal heights are reported as mean ± SEM, and differences between the two experimental groups analysed using an unpaired two-tailed Student's t test. Due to variation in the permeability data, clearance half times are expressed as median values together with upper and lower quartiles, and differences between groups analysed using the Mann-Whitney U test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Lung Permeability

The changes in the permeability of the alveolar epithelium seen during this experiment are shown in Table I. There is no difference in the median clearance half-times between the endotoxin treated and saline treated groups in the period before smoke exposure (endotoxin alone $t_{1/2}$ = 136 min., saline alone $t_{1/2}$ = 140 min., p = 0.95). Cigarette smoke leads to an increase in permeability within minutes of exposure, which is significantly greater in endotoxin-primed animals than in controls (saline/smoke $t_{1/2}$ = 54 min., endotoxin/smoke $t_{1/2}$ = 24 min., p = 0.0027).

ESR Spectroscopy

Analysis of the plasma samples from the six saline/smoke experiments either before or after smoke exposure did not demonstrate the presence of any spin adducts to the

TABLE I
Clearance half times of ^{99m}Tc-DTPA from the lungs (minutes, median with 25–75% interquartile range).

	Clearance half times
Saline	140 (102–220)
Endotoxin	136 (133–145)
Saline/smoke	54 (35–62)
Endotoxin/smoke	25 (20–28)†

† Endotoxin/smoke half time significantly smaller than saline/smoke, p = 0.0027, Mann-Whitney U test.

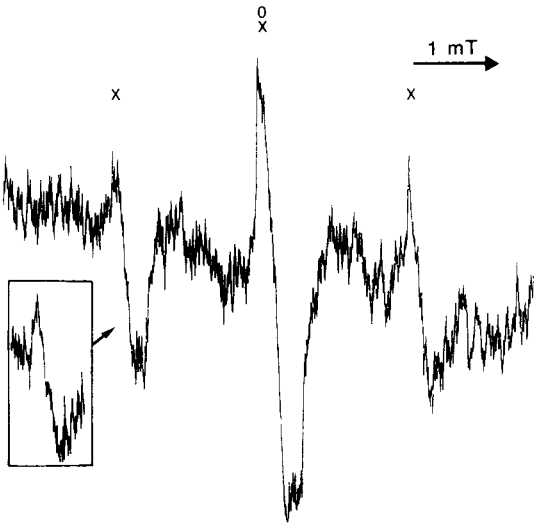


FIGURE 2 Electron spin resonance spectrum of a plasma sample obtained from an endotoxin-primed rabbit 1 minute after initiation of smoke inhalation. Spectrum assigned to a mixture of the signals from the ascorbyl radical (O) and a PBN spin adduct (x). Under high resolution conditions both the ascorbyl radical signal and the spin adduct signal show further doublet splittings (see inset for PBN adduct signal). ESR hyperfine coupling constants for spin adduct are approximately a_N 1.60, a_H 0.14 mT.

spin trap, though in all cases a relatively strong signal from the well characterised ascorbyl (vitamin C) radical was observed.

These results are in contrast with those obtained from the endotoxin/smoke series, in which although no signal was detected (apart from that due to the ascorbyl radical) before smoke administration, a signal assignable to an adduct to the spin trap was seen immediately after smoke inhalation (Figure 2). The spectrum shown in Figure 2 is typical of that due to a mixture of the spin adduct and the ascorbyl radical, in which a central broad line results from the overlapping of the spectral lines of the ascorbyl and spin adduct radicals. This line can be resolved, under high resolution conditions, into the well characterised doublet splitting from the ascorbyl radical and a further broad doublet. The outermost lines from the spin adduct can also be shown to consist of poorly resolved doublets under high resolution conditions. The parameters of the spin adduct signal of approximately a_N 1.60, a_H 0.14 mT are consistent[14] with the spin trapping by PBN of either a heteroatom-centred (possibly oxygen-derived) radical or a carbon-centred radical species which is highly electron deficient, although further speculation as to the exact nature of this adduct is not warranted at the present time on the basis of these results.

This adduct species was, in general, only observed during the first few minutes after smoke inhalation and no further species were observed at longer time points. As the intensity of the ESR signal is directly proportional to the free radical concentration, a time course of spin adduct formation can be constructed (Figure 3) for each animal. In all six experiments a peak in radical concentration (as measured by signal heights) is seen within the first ten minutes following smoke exposure, although the exact timing of the peak varied. In five out of the six cases the appearance of the adduct

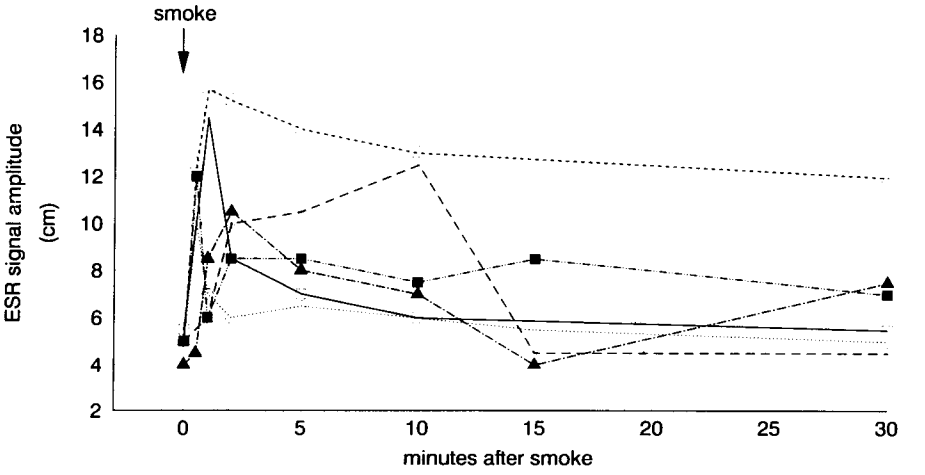


FIGURE 3 Smoke induced radical production in endotoxin primed rabbits. The spin trap adduct signal is expressed in terms of the peak to peak signal amplitude, and is uncorrected for baseline signal noise. Data from consecutive measurements in each animal.

signal was temporary; in the other case the signal persisted throughout the 30 min. over which the samples were taken. The results from all animals are collected in Figure 4, which demonstrates that this burst of radical production is statistically significant when compared to the control samples over the first ten minutes after smoke inhalation. These results suggest that immediately preceding the observation of

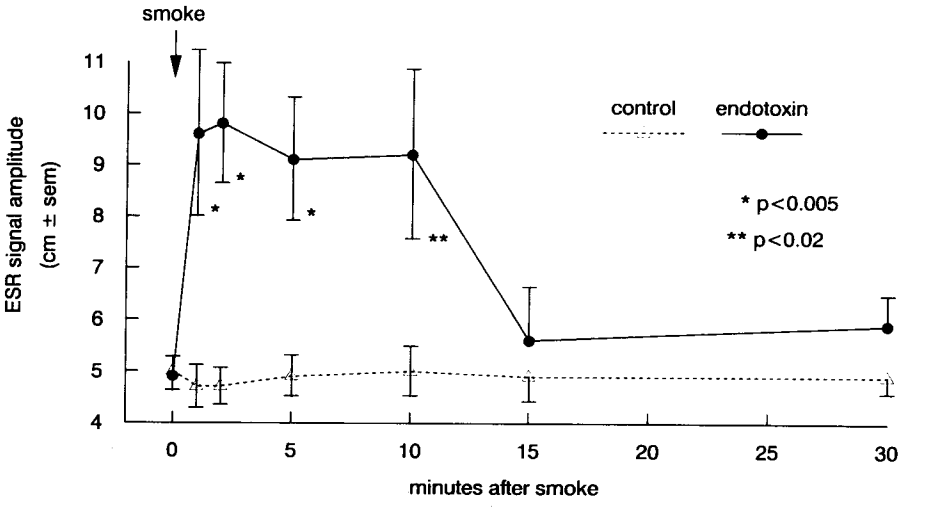


FIGURE 4 The time course of smoke induced radical generation in saline and endotoxin pretreated rabbits. The plasma spin trap adduct signal is expressed in terms of the mean peak to peak ESR signal amplitude for a given time, ± standard error of the mean (SEM), and is not corrected for the height of the baseline signal noise. The mean signal height in the endotoxin group is significantly greater than the (baseline noise) signal in the saline/smoke group at 1, 2, 5, and 10 minutes after smoke.

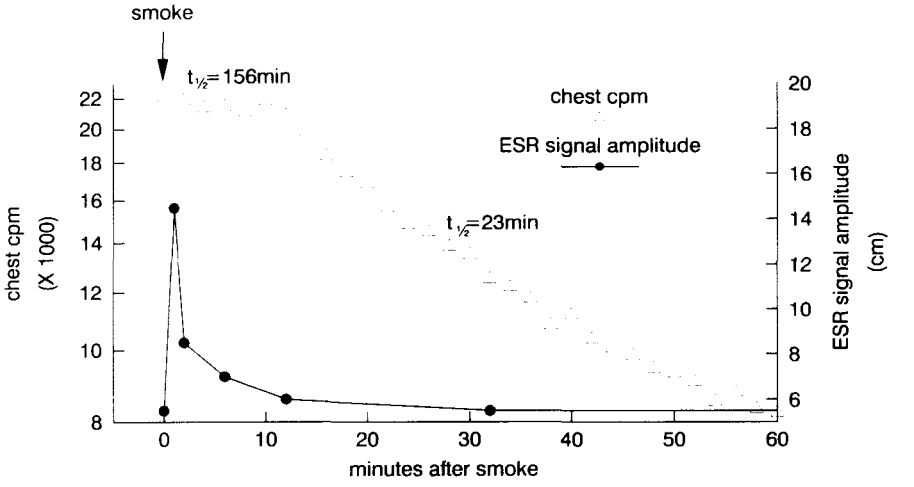


FIGURE 5 The temporal relationship between smoke induced increase in lung permeability and free radical production in an endotoxin pretreated rabbit. Radioactivity from ^{99m}Tc-DTPA, after aerosol delivery to the lungs, is recorded from the chest and expressed as counts per minute (cpm); lung permeability to this radiotracer is described in terms of the clearance half time, $t_{1/2}$ (in minutes). The spin trap adduct signal is expressed as in Figure 2. Data from a single experiment.

the spin adducts in the plasma, there must have been a burst of radical formation in the lung tissue.

Figure 5 demonstrates the temporal relationship between observation of the spin adducts and changes in alveolar epithelial permeability from a single endotoxin/smoke experiment. Exposure to smoke results in a burst of radical generation which subsides over the course of the next ten minutes, and is followed by a marked increase in lung permeability. These results suggest that the radical species trapped in these experiments are not merely a *consequence* of cellular damage by some other injurious process, but may be a *causative* factor.

DISCUSSION

There is considerable experimental evidence to suggest that the lungs are susceptible to radical mediated injury. Superoxide generating systems have been shown to cause acute lung injury in both isolated organ[20] and *in vivo* models,[21] although derivatives of superoxide such as the hydroxyl radical may be the harmful species.[4] The principal source of these radicals appears to be activated cellular elements of the inflammatory response,[22,23] although in the clinical setting the importance of hyperoxic lung injury cannot be ignored. Phorbol myristate acetate (PMA) is a potent white cell activator, and *in vivo* has been shown to cause damage to both the endothelial[24] and epithelial[25] components of the alveolar capillary barrier, the former at least being white cell dependent.[24] Since free radical scavengers protect the lungs against such injury[26] there is some reason to believe that white cells are in some way involved in the generation of radicals responsible for the lung injury seen in these preparations.

Endotoxaemia appears to be a particularly important event in the development of acute lung injury, and in humans severe sepsis is a major cause of ARDS.[3] Endotoxin infusions in sheep generate acute lung injury which is associated with increased tissue malondialdehyde content[27] and which is attenuated with antioxidant therapy.[28] The effect of endotoxin on the inflammatory response may therefore be of considerable consequence. Thus, endotoxin promotes the accumulation of neutrophils within the pulmonary microcirculation,[29] and in addition primes these cells, so that in response to a second stimulus (such as cigarette smoke) they become highly metabolically active and cytotoxic as they generate oxygen-derived free radicals (via the respiratory burst) and liberate potent proteolytic enzymes. Although acute lung injury may also develop through neutrophil independent mechanisms, an important experimental and clinical mechanism of lung injury would appear to be through the generation of oxygen-derived free radicals from endotoxin primed/activated neutrophils.

In this study we have confirmed the observation that pretreatment with endotoxin amplifies smoke induced damage to the alveolar epithelium, and have associated this enhanced effect with clear evidence for the generation of radicals immediately preceding and during the evolution of this damage. Thus we have demonstrated that while neither endotoxin nor smoke alone are able to generate sufficient plasma adduct signal to be detectable in our system, the combination of the two generates a burst of radical production that is associated with an immediate increase in the permeability of the alveolar epithelium.

The findings in the saline/smoke group require some discussion. Brief smoke exposure leads to an increase in permeability of the alveolar epithelium to ^{99m}Tc-DTPA, although it is not known whether the oxidant burden which smoke inhalation imposes on the lungs[30] is responsible for this injury. We were unable to detect systemic evidence of adduct formation in this experimental group, although the inherent sensitivity and operating conditions of the ESR spectrometer were such that low concentrations would not have been observed. Similarly, although endotoxin alone was not sufficient to generate any significant adduct signal, the generation of low concentrations could not be excluded.

It is tempting to hypothesise that this endotoxin/smoke model of lung injury demonstrates the effect of initial endotoxin priming on the subsequent response of white cells to an appropriate stimulus. Within the limits imposed by the sensitivity of the analysis, the results of this study are in support of the hypothesis that endotoxin promotes the accumulation of a population of primed white cells within the lung which then triggered, in this system by cigarette smoke, are able to generate a burst of radicals which result in tissue damage. Further work is required to confirm this attractive hypothesis.

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References

1. M.P. Barrowcliffe and J.G. Jones (1988) The pathophysiology and treatment of the adult respiratory distress syndrome (ARDS). In *Anaesthesia Review 5* (L. Kaufman Ed.). Churchill Livingstone. Edinburgh, pp. 182–195.

2. J.P. Wiener-Kronish, M.A. Gropper and M.A. Matthay (1990) The Adult Respiratory Distress Syndrome: definition and prognosis, pathogenesis and treatment. *British Journal of Anaesthesia*, **65**, 107–129.
3. A.A. Fowler, R.F. Hamman, J.T. Good, K.N. Benson, M. Batrd, D.J. Eberle, T.L. Petty and T.M. Hyers (1983) Adult respiratory distress syndrome: risk with common predispositions. *Annals of Internal Medicine*, **99**, 293–298.
4. J.E. Heffner and J.E. Repine (1989) Pulmonary Strategies of Antioxidant Defense. *American Review of Respiratory Disease*, **140**, 531–554.
5. K.L. Brigham and B. Meyrick (1984) Interactions of granulocytes within the lungs. *Circulation Research*, **54**, 623–635.
6. R.M. Jackson, A.L. Brannen, C.F. Veal and J.D. Fulmer (1988) Superoxide dismutase and cytochrome oxidase in collapsed lungs: possible role in reexpansion edema. *Journal of Applied Physiology*, **65**, 235–241.
7. M.P. Barrowcliffe and J.G. Jones (1987) Solute permeability of the alveolar-capillary barrier. *Thorax*, **42**, 1–10.
8. H.O'Brodovitch, G. Coates, J. Kay, D. Muysson (1989) Simultaneous measurement of lung clearances in normal and damaged lungs. *Journal of Applied Physiology*, **66**, 2293–2297.
9. N.C. Staub, R.W. Hyde and E. Crandall (1990) Workshop on Techniques to Evaluate Lung Alveolar-Microvascular Injury. *American Review of Respiratory Disease*, **141**, 1071–1077.
10. R. Bolli, B.S. Patel, M.O. Jeroudi, E.K. Lai and P.B. McCay (1988) Demonstration of Free Radical Generation in "Stunned" Myocardium of Intact Dogs with the Use of the Spin Trap α-Phenyl *N-Tert*-Butyl Nitrone. *Journal of Clinical Investigation*, **82**, 476–485.
11. M.J. Green, H.A.O. Hill and D.G. Tew (1985) Applications of spin-trapping to biological systems. *Biochemical Society Transactions*, **13**, 600–603.
12. J.S. Smit, D. Myers, B.G. Gough and J.G. Jones (1989) Synergism in acute lung injury. *British Journal of Anaesthesia*, **63**, 632–633.
13. P.G. Murphy, D. Myers and J.G. Jones (1990) Oxpentifylline attenuates endotoxin/smoke induced lung injury. *British Journal of Anaesthesia*, **65**, 578–579.
14. G.R. Buettner (1987) Spin trapping: ESR parameters of spin adducts, *Free Radical Biology and Medicine*, **3**, 259–303.
15. E.K. Lai, C. Crossley, R. Sridhar, H.P. Misra, E.G. Janzen and P.B. McCay (1986) *In vivo* spin trapping of free radicals generated in brain, spleen and liver during gamma-irradiation in mice, *Archives of Biochemistry and Biophysics*, **224**, 156–160.
16. M.J. Davies (1987) Application of electron spin resonance spectroscopy to the identification of radicals produced during lipid peroxidation, *Chemistry and Physics of Lipids*, **44**, 149–173.
17. H.D. Connor, R.G. Thurman, M.D. Galizi and R.P. Mason (1986) The formation of a novel free radical metabolite from CCl_4 in the reperfused rat liver *in vivo*, *Journal of Biological Chemistry*, **261**, 4542–4548.
18. M.P. Barrowcliffe, C. Otto and J.G. Jones (1988) Pulmonary clearance of ^{99m}Tc-DTPA: influence of background activity. *Journal of Applied Physiology*, **64**, 1045–1049.
19. H.O'Brodovitch and G. Coates (1987) Pulmonary Clearance of ^{99m}Tc-DTPA: A Noninvasive Assessment of Epithelial Integrity. *Lung*, **165**, 1–16.
20. R.M. Tate, K.M. Vanbenthuysen, D.M. Shasby, I.F. McMurty and J.E. Repine (1982) Oxygen radical mediated pulmonary edema and vasoconstriction in isolated perfused rabbit lungs. *American Review of Respiratory Disease*, **126**, 802–806.
21. K.J. Johnson, J.C. Fantone III, J. Kaplan and P.A. Ward (1981) *In vivo* damage of rat lungs by oxygen metabolites. *Journal of Clinical Investigation*, **67**, 983–993.
22. S.J. Weiss (1989) Tissue destruction by neutrophils. *New England Journal of Medicine*, **320**, 365–376.
23. J.E. Repine (1985) Oxygen derived free radicals, Neutrophils and the lung. In *Acute Respiratory Failure* (W.M. Zapol and K.J. Falks Eds.). Marcel Dekker. New York. pp. 347–378.
24. R.M. Tate, K.M. Vanbenthuysen, D.M. Shasby, I.F. McMurty and J.E. Repine (1982) Granulocytes mediate acute edematous lung injury in rabbits and in isolated rabbit lungs perfused with phorbol myristate acetate. *American Review of Respiratory Disease*, **125**, 443–447.
25. P.G. Murphy, D.S. Myers, M. Green and J.G. Jones (1991) Ultrastructural evidence for epithelial injury in experimental acute lung injury. *British Journal of Anaesthesia*, **66**, 398.
26. R.C. Allison, E.M. Hernandez, V.R. Prassad, M.B. Grisham and A.E. Taylor (1988) Protective effects of O_2 radical scavengers and adenosine in PMA-induced lung injury. *Journal of Applied Physiology*, **64**, 2175–2182.
27. R.H. Demling, C. Lalonde, L.J. Jin, P. Ryan and R. Fox (1986) Endotoxaemia causes increased lung tissue lipid peroxidation in unanaesthetised sheep. *Journal of Applied Physiology*. **60**, 2094–2100.

28. N.C. Olson, D.L. Anderson and M.K. Grizzle (1987) Dimethylthiourea attenuates endotoxin induced acute respiratory failure in pigs. *Journal of Applied Respiratory Physiology*, **63**, 2426–2432.
29. K.L. Brigham and B. Meyrick (1985) Endotoxin and lung injury. *American Review of Respiratory Disease*, **133**, 913–927.
30. W.A. Pryor, M.M. Dooley and D.F. Church (1986) The mechanisms of the inactivation of human alpha-1-proteinase inhibitor by gas-phase cigarette smoke. *Advances in Free Radical Biology and Medicine*, **2**, 161–188.

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