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N-ACETYLCYSTEINE AMELIORATES HYPEROXIC LUNG INJURY IN THE PRETERM GUINEA PIG

SIMON C. LANGLEY* and FRANK J. KELLY
Cardiovascular Research, The Rayne Institute, St Thomas' Hospital, London, U.K.

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Abstract—The therapeutic efficacy of *N*-acetylcysteine (NAC) in the management of hyperoxia-induced lung injury was assessed using the preterm guinea pig model of prematurity. Preterm guinea pig pups were delivered by Caesarean section 3 days preterm, and exposed to either 21 or 95% oxygen for 72 hr. NAC (200 mg/kg body weight) or saline was injected twice daily. Bronchoalveolar lavage fluid (BALF) from hyperoxia-exposed pups contained significantly higher protein concentrations and an increased number of neutrophils. NAC partly ameliorated lung injury, preventing the increase in BALF protein concentration, which is generally associated with oedema. There was no effect on the movement of neutrophils into the lung airspaces in response to oxygen. Treatment with NAC had no effect on lung or liver glutathione (reduced) (GSH) concentrations either after 2 hr post-administration, or over the full 72 hr experimental period. An apparent resistance of the lung to increased synthesis or uptake of GSH was demonstrated by the lack of effect of direct administration of GSH, its isopropyl ester or 2-oxo-4-thiazolidine carboxylic acid. Oxygen exposure alone (95%) increased lung concentrations by 60–70%. It would, therefore, appear from this data that NAC may have potential as a future component of antioxidant therapy, although its effects are not mediated through increased GSH levels.

Premature infants born between 24 and 32 weeks gestation frequently suffer oxygen free radical-mediated pulmonary injury as a consequence of the ventilator therapy they receive [1, 2]. In a significant proportion of these infants this acute injury develops into bronchopulmonary dysplasia (chronic lung disease) which may be fatal, or lead to long periods of hospitalization and, potentially, respiratory problems throughout childhood [2, 3].

Due to the ethical and practical difficulties associated with the study of premature infants, a number of animal models have been developed. Of these, one of the most suitable small animal models is the preterm guinea pig [4–8]. The preterm guinea pig lung is both morphologically and biochemically immature [7, 8], as in the preterm human infant. Moreover, the injury sustained in hyperoxic conditions is similar to that observed in premature babies undergoing oxygen therapy [6].

Parallel to studies aimed at understanding the mechanisms leading to acute lung injury and the transition to chronic lung disease, a number of agents have been applied to the guinea pig and other models, with a view to developing an appropriate programme of antioxidant therapy. To date, these approaches have included the administration of the antioxidant enzymes superoxide dismutase and catalase (CAT†) [9–11], and the free radical scavenger vitamin E [12].

* Corresponding author: Dr S. C. Langley, Department of Human Nutrition, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K. Tel. (0703) 594320; FAX (0703) 594459.

† Abbreviations: BALF, bronchoalveolar lavage fluid; CAT, catalase; GSH, glutathione (reduced); GPx, glutathione peroxidase; NAC, *N*-acetylcysteine; OTC, 2-oxo-4-thiazolidine carboxylic acid.

N-Acetylcysteine (NAC) is a drug which has received considerable attention in recent years. It is used primarily as a mucolytic agent in the management of bronchitis [13] and in the treatment of acetaminophen overdose [14]. As such, the pharmacokinetics of this thiol compound are well understood [14, 15]. NAC has the capacity to react directly with electrophiles, and thus has antioxidant and cytoprotective potential. NAC is readily deacylated to form thiol metabolites, including cysteine and glutathione (GSH) [16, 17]. GSH is itself a potent antioxidant, believed to play a major role in the protection of the lung from oxidative injury [17–20]. Oral administration of NAC has been shown to elevate circulating GSH concentrations in humans [17–19], whilst rat lung perfusion of previously GSH-depleted tissues with NAC partially restored GSH levels [17].

The efficacy of NAC as a protective agent against free radical-induced lung injury has been previously demonstrated using cultured bronchial fibroblasts [17], sheep [18] and adult rats [19, 20]. In the present study we have assessed the benefit of NAC in the prevention of acute oxygen-induced lung injury, using the preterm guinea pig model.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were purchased from the Sigma Chemical Co. (Poole, U.K.) or BDH (Poole, U.K.) unless stated otherwise in the text. YM737 was a kind gift of Yamanouchi Pharmaceuticals Ltd (Japan).

Animals. Virgin female Dunkin–Hartley guinea pigs (550 g) were caged in pairs in a temperature-controlled room (22–24°) on a 12 hr light : 12 hr dark cycle. Animals had free access to food and water. Timed pregnancies were established as described

previously [6]. On day 65 of gestation, pups were delivered by Caesarean section, under Fluothane anaesthesia [6]. All pups were delivered within 5 min of the initial anaesthetic administration. The mothers were killed by exsanguination.

Pups were housed with lactating surrogate mothers in 25 L capacity purpose-built plastic boxes with wire mesh bottoms over sterile sawdust. Cages were washed and disinfected daily. Each litter was divided and pups were allocated to cages into which was fed room air (21% oxygen) or 95% oxygen. This concentration was achieved by passing compressed air and 100% oxygen through pressure-reducing regulators and flow meters. Previous studies have established that humidity in the cages does not differ significantly between 21% and 95% oxygen regimes. Oxygen concentrations were verified using a Servomex oxygen analyser (Crowborough, Sussex, U.K.). Gases entered the cages at a rate of 3.5 L/min.

Experimental protocol. Following delivery, pups from a total of 20 litters were randomly distributed into four groups which were treated as follows: 21% O_2, saline injected (N = 15); 21% O_2, NAC injected (N = 18); 95% O_2, saline injected (N = 17); 95% O_2, NAC injected (N = 23). NAC was administered i.p. at a dose of 400 mg/kg body weight divided into two daily 0.2 mL injections given at 9.00 a.m. and 4.30 p.m. daily. Control animals received 0.2 mL saline i.p. at these times. At the end of the 72 hr experimental period the pups were anaesthetized and bronchoalveolar lavage was performed [6]. Blood was taken by heart puncture into heparinized tubes. The abdominal aorta was cut and the lungs perfused by injecting 10 mL of 0.9% saline into the right ventricle of the heart. Lung and liver (100–200 mg portions) were removed for GSH determination, before the remainder of the lungs were removed and immediately frozen in liquid nitrogen. All tissues were stored for up to 2 months at −80° prior to assay of antioxidant enzymes.

A further group of eight preterm pups were administered NAC, at the above dose at birth, but killed 1 or 2 hr later to determine the efficacy of NAC as an inducer of GSH synthesis. Pulmonary and hepatic GSH levels in these pups were compared to those of four pups killed immediately following delivery.

In order to assess the response of the lung to putative enhancers of tissue GSH status, the effects of GSH, YM737, a GSH isopropyl ester, and 2-oxo-4-thiazolidine carboxylic acid (OTC) were assessed in term pups aged 1–3 days. All these agents were administered i.p. GSH was given at a dose of 1 mmol/kg, YM737 at 1 g/kg and OTC at 100 mg/kg. Control pups were injected with the appropriate volume of saline. The animals were killed 2 hr following drug administration, and pulmonary and hepatic GSH determined.

Bronchoalveolar lavage and leucocyte counting. Pups were anaesthetized with 50 mg/kg body weight sodium pentobarbital (i.p.), and bronchoalveolar lavage performed with five 2 mL aliquots of sterile 0.9% saline [6]. Typically recovery of lavage fluid was 80–95%.

Total leucocyte numbers in bronchoalveolar lavage fluid (BALF) were counted using a Neubauer haemocytometer. Cytospin preparations of BALF were stained with May and Grunwalds and Giemsa reagents. Differential cell counts were performed in 300 cells.

Total protein concentrations in BALF were determined by the bicinchoninic acid method [21]. Absorbance at 560 nm was measured using a Biotek EL340 microplate reader, using a bovine serum albumin standard.

Determination of glutathione. Glutathione was determined by the method of Tietze [22]. Lung and liver samples were homogenized in 3 mL 0.2 N perchloric acid and centrifuged at 2000 rpm for 10 min. BALF was centrifuged at 1000 rpm for 10 min to remove the cells, and was assayed undiluted. Oxidized glutathione was assayed by conjugating GSH with vinylpyridine. Oxidized glutathione levels accounted for less than 5% of the total glutathione measured, and was frequently not detectable. Oxygen exposure or NAC treatment did not alter oxidized glutathione levels, and these measurements are therefore not reported.

Determination of antioxidant enzyme activities. Lung samples (400 mg) were prepared as previously described [7] and frozen at −80° before analysis of antioxidant enzyme activity. Following preparation, 80–90% of activity in the homogenate is typically recovered (Kelly *et al.*, unpublished observations).

Glutathione peroxidase (GPx) activity was assayed by the method of Beutler [23] adapted to a Biotek microplate reader. CAT activity was assayed by the method of Aebi [24], using bovine liver catalase standards.

All enzyme activities were expressed per mg DNA. DNA was assayed by the method of Richards [25].

Statistical analysis. The results shown were obtained from a single study. Data were analysed using a 2-way analysis of variance (ANOVA) for an

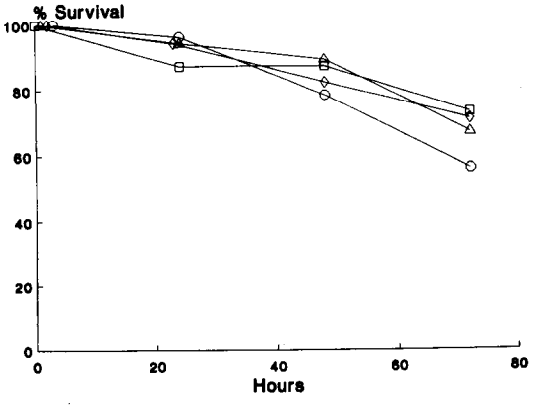

Fig. 1. The effect of NAC and oxygen exposure on the survival of preterm guinea pigs over 72 hr. Preterm guinea pig pups were exposed to 21% or 95% oxygen for 72 hr and were injected with either 200 mg/kg i.p. NAC or saline twice daily. Air:saline (□); air:NAC (△); oxygen:saline (◇); oxygen:NAC (○).

Table 1. The effect of NAC administration on BALF protein content and inflammatory cell numbers

Treatment	Protein (mg/mL)	Total leucocytes ($\times 10^6$ cells/mL)	Neutrophils ($\times 10^6$ cells/mL)
21% oxygen			
Saline	0.30 ± 0.03	0.44 ± 0.08	0.0093 ± 0.0046
NAC	0.34 ± 0.06	0.42 ± 0.07	0.0040 ± 0.0010
95% oxygen			
Saline	0.52 ± 0.06*	0.87 ± 0.25	0.0190 ± 0.0050*
NAC	0.34 ± 0.03	0.72 ± 0.20	0.0110 ± 0.0020*

Preterm guinea pig pups were exposed to 21% or 95% oxygen for 72 hr and were injected with either 200 mg/kg i.p. NAC or saline twice daily. Data are presented as means ± SEM.

* Indicates a significant effect of oxygen $P < 0.05$ or better.

ANOVA indicates a significant effect of oxygen on BALF protein ($F = 6.17$ df 1,49) and on neutrophil numbers ($F = 5.87$ df 1,25). NAC and oxygen had an interactive effect on BALF protein content only ($F = 6.08$ df 1,44).

Table 2. The effect of NAC administration on pulmonary activities of CAT and GPx

Treatment	CAT (kU/mg DNA)	GPx (U/mg DNA)
21% oxygen		
Saline	99.70 ± 10.23	1.08 ± 0.22
NAC	76.09 ± 14.54	0.95 ± 0.15
95% oxygen		
Saline	150.29 ± 24.89	1.31 ± 0.17
NAC	97.04 ± 10.32	0.88 ± 0.10

Preterm guinea pig pups were exposed to 21% or 95% oxygen for 72 hr and were injected with either 200 mg/kg i.p. NAC or saline twice daily.

Data are presented as means ± SEM.

unbalanced design. Where significant interactions were indicated statistical probability was determined using Student's *t*-test. Survival curves were compared using the Mantel–Haenszel test. A probability value of less than 5% was taken as significant.

RESULTS

The relative survival rates of the animals in each of the four groups is shown in Fig. 1. No significant differences in survival were observed, although there was some indication of toxicity using NAC at the given concentration (six deaths from 18 NAC:air vs 4/15 saline:air, 10/23 NAC:oxygen vs 5/17 saline:oxygen).

Lung injury, as indicated by BALF protein concentration, was significantly diminished by NAC treatment (Table 1). Exposure to 95% oxygen for 72 hr significantly elevated BALF protein levels in saline-injected pups, whilst in NAC-treated animals no oxygen-induced increase in protein concentration was observed. BALF leucocyte numbers were unaltered by oxygen exposure or NAC administration (Table 1). Neutrophil numbers increased significantly in oxygen-exposed animals (Table 1), regardless of NAC treatment. The activities of the antioxidant enzymes GPx and CAT were unaltered by NAC administration or oxygen exposure (Table 2).

Glutathione concentrations in tissues and in BALF are shown in Table 3. BALF and liver GSH were unaltered by either oxygen exposure or NAC treatment. Lung GSH concentrations increased significantly in response to oxygen exposure (63% saline:oxygen, 70% NAC:oxygen). NAC treatment did not alter the pulmonary GSH level in either air or oxygen.

The capacity of the lung and liver to synthesize GSH or take up exogenous GSH in response to drugs, was assessed in 3-day-old term neonates. Direct administration of GSH (Table 4) failed to mediate an increase in pulmonary GSH concentrations 2 hr post-injection. Hepatic levels of the tripeptide were significantly elevated (78%) by GSH treatment. The effects of other putative enhancers of GSH status, in 1-day-old pups, over the same timescale are shown in Table 5. Glutathione isopropyl ester (YM737) had no effect in lung, but elevated hepatic GSH levels by more than 2-fold. OTC (100 mg/kg) failed to alter GSH concentrations in either tissue. Similarly, the administration of NAC to preterm guinea pig pups immediately following delivery by Caesarean section, failed to increase either pulmonary or hepatic GSH concentrations by 1 or 2 hr post-injection (Fig. 2).

DISCUSSION

NAC has been described as "a drug with an interesting past and a fascinating future" [13]. NAC is currently used clinically in the management of respiratory problems [13], and treatment of drug overdose [14]. A wide variety of new uses have been proposed, including the treatment of oxidative lung injury [26], and other conditions that are believed to be free radical mediated, for example rheumatoid arthritis [13]. Oxidative lung injury is most frequently

Table 3. The effect of NAC administration on tissue and BALF GSH concentrations

Treatment	GSH concentration Lung (μmol/g tissue)	Liver (μmol/g tissue)	BALF (μM)
21% oxygen			
Saline	0.83 ± 0.09	4.04 ± 0.99	1.14 ± 0.59
NAC	0.70 ± 0.05	3.01 ± 0.37	3.03 ± 1.82
95% oxygen			
Saline	1.35 ± 0.17*	3.89 ± 0.86	0.20 ± 0.09
NAC	1.19 ± 0.16*	6.50 ± 2.45	2.70 ± 1.70

Preterm guinea pig pups were exposed to 21% or 95% oxygen for 72 hr and were injected with either 200 mg/kg i.p. NAC or saline twice daily.

Data are presented as means ± SEM.

* Indicates a significant effect of oxygen $P < 0.005$, $F = 13.75$ df 1,37.

Table 4. The effect of GSH administration on pulmonary and hepatic GSH concentrations

Treatment	N	GSH (μmol/g tissue) Lung	Liver
Saline	4	2.02 ± 0.20	4.35 ± 1.03
GSH	4	2.14 ± 0.28	7.76 ± 0.30*

Three-day-old term neonatal guinea pig pups were injected with either 1 mmol/kg i.p. GSH or saline and killed 2 hr later.

Data are presented as means ± SEM.

* Indicates a significant effect of GSH treatment $P < 0.05$.

Table 5. The effect of YM737 or OTC administration on pulmonary and hepatic GSH concentrations

Treatment	N	GSH (μmol/g tissue) Lung	Liver
Saline	4	1.00 ± 0.08	3.21 ± 0.21
YM737	6	1.08 ± 0.08	7.72 ± 1.19*
OTC	4	0.79 ± 0.07	4.01 ± 0.07

One-day-old term neonatal guinea pig pups were injected with 1 mmol/kg i.p. GSH, or 1 g/kg YM737, or 100 mg/kg OTC, controls receiving an appropriate volume of saline and killed 2 hr later.

Data are presented as means ± SEM.

* Indicates a significant effect of YM737 treatment $P < 0.05$.

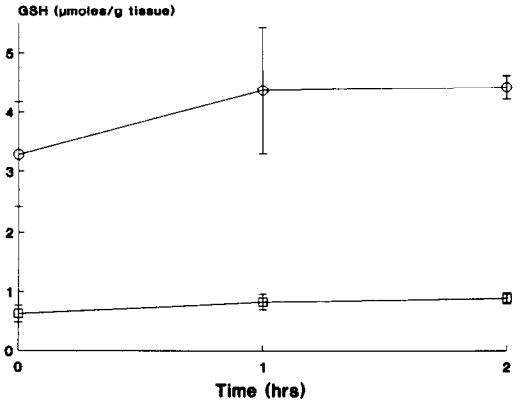

Fig. 2. The effect of NAC administration on pulmonary and hepatic GSH concentrations. Preterm guinea pig pups were injected with 200 mg/kg i.p. NAC immediately following delivery by Caesarean section and killed 1 or 2 hr later. Data are presented as means ± SEM. A group of pups were killed at delivery for use as zero time controls. Lung GSH (□); liver GSH (○).

encountered in premature babies in special care units. In the present study we assessed the potential therapeutic benefit of NAC to such infants, using the preterm guinea pig model of oxygen-induced pulmonary damage. This initial study has shown that NAC does indeed ameliorate oxidative injury to the immature lung.

Many of the effects attributed to NAC have been proposed to be mediated through an increase in tissue or circulating levels of GSH [17, 26]. Pulmonary GSH depletion by tobacco smoke is partially blocked by NAC administration [17], and prolonged oral administration of NAC leads to a sustained increase in circulating GSH levels [26], with rapid onset [17, 26]. More recently, however, Cotgreave *et al.* [16] have suggested that NAC is a relatively poor precursor for GSH synthesis, and that any observed antioxidant or cytoprotective effects are more likely to be mediated by direct free radical scavenging or other mechanisms. The results of the present study are consistent with this hypothesis. Administration of NAC failed to stimulate pulmonary GSH synthesis in the short term (i.e. 1–2 hr post-injection, which is the time of peak NAC concentrations in plasma [17, 26]). Likewise, no elevation of GSH concentration was observed over a longer period and, indeed, would be unlikely as NAC has a low bioavailability (less than 10%) [14, 15, 17], due to

extensive first pass metabolism and the formation of disulphides in circulation. NAC is rapidly turned over *in vivo* [14, 15], with total clearance being observed 3 hr following oral administration [17]. The primary mechanism of NAC clearance is deacylation, with associated increases in plasma cysteine concentrations [17]. Under appropriate conditions cysteine may be stored in the liver as GSH [27], but in the preterm guinea pig, the requirement for protein synthesis to satisfy the rapid growth demand may remove much of any cysteine released from NAC.

GSH concentrations in tissues are notoriously difficult to manipulate therapeutically, without prior depletion. In the present study the administration of GSH, its isopropyl ester YM737, NAC and OTC failed to increase pulmonary GSH levels in term guinea pig neonates, although transient increases in the liver were elicited by GSH and YM737. Direct intratracheal administration of GSH or YM737 has been shown to decrease GSH concentrations in the lung (S. C. Langley, unpublished observation). This apparent resistance of the lung to exogenous stimulation of GSH synthesis or the uptake of exogenously supplied GSH, suggests that it is unlikely any antioxidant protection to the lung could be mediated via NAC-induced increases in GSH levels. Exposure of animals to oxygen did, however, elevate pulmonary GSH concentrations, suggesting that free radical damage may stimulate GSH synthesis. As this oxygen-induced elevation of GSH concentrations did not provide protection against lung injury in saline-treated controls, it is assumed to be initiated only after the onset of damage. Such a response to oxygen has not previously been reported in this animal model.

There was no effect of NAC on the relative survival of the pups in oxygen, but other indicators of oxygen toxicity were altered. The preterm guinea pig, like the human infant, undergoes an inflammatory response in the lung when exposed to oxygen. This response is characterized primarily by an influx of neutrophils into the lung airspaces and interstitium [6]. An increase in vascular permeability is also observed, and is measured as an increase in BALF protein concentrations [6]. Approximately 50% of this protein has been shown to be serum albumin. This increased permeability ultimately leads to pronounced pulmonary oedema, which is a major cause of death in these animals. NAC treatment failed to ameliorate the inflammatory response, but successfully prevented the increase in vascular permeability.

The unabated influx of neutrophils in NAC-treated animals exposed to oxygen indicates that some degree of lung damage may still be occurring, in the absence of oedema. The role of the neutrophil in lung injury of this type is an unresolved issue, and the question of whether neutrophils cause injury, or are merely sequestered in response to injury for a possible reparative function remains open.

Pulmonary capillary endothelial cells are believed to be among the first to undergo oxidative damage and destruction on exposure of the lungs to hyperoxic conditions [28]. It is this breakdown of capillary integrity that allows the influx of fluid into the airspaces, leading to oedema. Critical damage may occur very early on in the period of oxygen exposure. Transient depletion of pulmonary GSH has been demonstrated to accelerate greatly the onset of measurable lung injury in term guinea pigs and death in preterm pups (S. C. Langley, unpublished observations). Future clinical usage of free radical scavengers may, therefore, need to be on a prophylactic basis, in order to cover the early phase during which damage may be initiated.

Whilst there was no significant effect of NAC on the concentrations of GSH in BALF, there was a tendency for BALF GSH concentrations to be increased in NAC-treated animals. It is therefore possible that an increased extracellular GSH pool may have a role in the attenuation of oxygen-induced lung injury by NAC. Alternatively, the possible site of action for NAC may be specifically at the level of pulmonary capillary endothelial cells, either as a direct scavenger of free radicals, or through breakdown to thiols capable of conjugating reactive species. Vascular endothelial cells are the primary sites of NAC deacylation [16]. As the bioavailability of NAC is low, the drug must be highly potent at low intracellular concentrations. The unaltered activities of GPx and CAT in NAC-treated animals would suggest that induction of antioxidant enzymes is not a mechanism of action, although induction of the superoxide dismutase activities cannot be excluded. Previously, in the preterm guinea pig model, induction of antioxidant enzymes over a 3–5 day period in response to 3 days hyperoxic exposure has been observed (Kelly *et al.*, unpublished observations). In the present study there were non-significant increases in both CAT and GPx activity in 95% oxygen, which were apparently prevented by NAC treatment. The trend for increased activity of CAT and GPx following 72 hr hyperoxia may be the beginning of an induction, which we have frequently found to reach statistical significance after 2 days recovery from the oxygen treatment. If enzyme induction is a response to lung injury, then these data may provide further evidence for the therapeutic benefit of NAC treatment.

The present study has, like others [17–20], demonstrated that NAC may be used as a component of future antioxidant therapies. Clearly there is a need to determine the appropriate dose regimen and route of administration, in addition to the time course and mechanism of action. The data presented do, however, suggest future potential for this drug in the treatment of preterm infants at risk of developing chronic lung disease.

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