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Depletion of Pulmonary Glutathione Using Diethylmaleic Acid Accelerates the Development of Oxygen-induced Lung Injury in Term and Preterm Guinea-pig Neonates

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Abstract—Dietary or chemical depletion of pulmonary glutathione in adult rats and mice, has been demonstrated to exacerbate the toxic effects of high oxygen concentrations. The present paper has examined this phenomenon in a guinea-pig model of prematurity, using the electrophilic agent diethylmaleic acid (DEM) to provide a transient (up to 12 h) pulmonary glutathione depletion. Full-term and 3-days preterm guinea-pig pups were studied to assess the possible role for glutathione deficiency as a mechanism mediating the increased susceptibility of the immature lung to oxygen free-radical damage. The administration of DEM to guinea-pig neonates depleted lung glutathione by 90% (term) or 68% (preterm) over 2 h. On exposure of pups to 95% oxygen for 48 h, DEM increased the incidence of oxygen-related death to 31% in term pups and 100% in preterm pups. Term pups exposed to hyperoxia and treated with DEM showed evidence of pulmonary injury, indicated by an influx of neutrophils into the lung airspaces, and elevated microvascular permeability. Control pups exposed to 95% oxygen were found to have uninjured lungs after 48 h. We conclude that glutathione is an essential component of the pulmonary antioxidant array in neonates. Glutathione may be of particular importance in the early phase of oxygen exposure. The deficiency of lung glutathione observed in preterm animals may account for their increased susceptibility to oxygen-induced pulmonary injury.

The biochemical and morphological maturation of the lung is a late gestational process (O'Brodovich & Mellins 1985), and consequently infants born prematurely, early in the last trimester of gestation, often develop respiratory distress syndrome; intervention with high concentrations of oxygen is often required for a successful clinical outcome. Oxygen therapy is itself associated with clinically significant pulmonary sequelae (Phelps 1982). Oxygen-centred free-radicals have the capacity to react with most cellular components, causing extensive tissue damage (Freeman & Crapo 1982). Infants undergoing long-term oxygen therapy can develop chronic lung disease, which often has a poor prognosis.

The most abundant non-enzymatic antioxidant is the tripeptide glutathione. Glutathione is the substrate for the antioxidant enzyme glutathione peroxidase, which detoxifies organic hydroperoxides and hydrogen peroxide (Jenkinson et al 1984). Glutathione also has the capacity to scavenge oxidizing species directly by virtue of its strongly reducing thiol group (Meister 1988), and has been postulated to be an important component of the defence against oxidative injury to the lung. Depletion of pulmonary glutathione, through the imposition of periods of fasting, has been demonstrated to increase oxygen-related mortality rates in adult rats and mice (Deneke et al 1983, 1985a; Smith et al 1990), with associated evidence of lung injury (Smith et al 1990). Short-term pulmonary glutathione depletion, using diethylmaleic acid (DEM), enhances oxygen toxicity in adult rats (Deneke et al 1985b, 1989; Weber et al 1990). Chronic depletion of glutathione using the inhibitor of glutathione synthesis, buthionine sulphoximine, leads to mitochondrial defects and morphological changes in the lungs of neonatal mice in normoxic conditions (Martensson et al 1989a), suggesting that glutathione may play a role in the maintenance of normal lung structure and function.

In the present study the effects of DEM have been assessed in guinea-pig neonates exposed to hyperoxic conditions. The biochemical and morphological development of the lungs are similar in guinea-pigs and man (Kelly et al 1991). The preterm guinea-pig pup exposed to high tensions of oxygen develops a lung injury that has similarities to that sustained by infants (Kelly et al 1991). Acute lung injury is characterized by an influx of inflammatory cells into the airspaces and interstitium of the lung, and a breakdown of capillary endothelial integrity (Massaro 1986). In order to assess the effects of glutathione on the immature lung, the duration of oxygen exposure was kept to 48 h, a time at which oxidative injury is minimal in this model (Kelly et al 1991).

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Materials and Methods

Chemicals

All chemicals and reagents were purchased from Sigma (Poole, UK) or BDH (Poole, UK) unless stated otherwise.

Animals

Virgin female Dunkin-Hartley guinea-pigs, 550 g, were caged in pairs in a temperature controlled room (22–24°C) on a 12-h light–12-h dark cycle. Animals had free access to food and water. Timed pregnancies were established as previously described (Kelly et al 1991) and allowed to proceed to full term (day 68) or pups were delivered prematurely, on day 65 of gestation by caesarian section (Kelly et al 1991). 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. Preterm pups have been previously demonstrated to feed normally and gain weight under these conditions of housing (Kelly et al 1991). 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. Oxygen concentrations were verified using a Servomex oxygen analyser (Crowborough, Sussex, UK). Gases entered the cages at a rate of 3·5 L min^{-1}.

Experimental protocol

Animals were randomly allocated to groups exposed to 21 or 95% oxygen. Within these groups the pups were further divided into groups to be administered 0·2 mL arachis oil (vehicle) or DEM (100 mg kg^{-1}), in a volume of 0·2 mL. Injections were administered by the intraperitoneal route. Each group contained 6–13 animals.

For time-course studies a single injection of DEM was administered, and animals killed at 30 min or 2, 4, 18, 24, and 48 h after the injection. Four animals were studied in each group. Animals allocated to the study of glutathione depletion and hyperoxic lung injury received injections at 0900 and 1500 h on the first day of the study.

Bronchoalveolar lavage and leucocyte counting

Pups were anaesthetized with 50 mg kg^{-1} sodium pentobarbitone (i.p.), and bronchoalveolar lavage performed with five 2-mL portions of sterile 0·9% NaCl (saline) (Kelly et al 1991). 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 on 300 cells.

Total protein concentrations in BALF were determined by the bicinchoninic acid method (Smith et al 1985). 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 (1969). Lung and liver samples were homogenized for 3 s in 0·2 M perchloric acid and centrifuged at 4000 *g* for 10 min at 4°C. BALF was centrifuged at 1000 rev min^{-1} for 10 min to remove the cells, and was assayed undiluted. Oxidized glutathione was assayed by conjugating glutathione with vinylpyridine (Griffith 1980). Oxygen exposure or DEM treatment did not alter oxidized glutathione levels, and these measurements are therefore not reported.

Statistical analysis

Data were analysed using a two-way analysis of variance for an 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

Depletion of glutathione by DEM

DEM-induced changes in lung glutathione concentrations were set against a shifting background. In term pups, pulmonary glutathione decreased by 37% relative to concentrations at birth, over the initial 24 h post-partum period. By 48 h, control glutathione concentrations were not significantly different from those at the start of the experiment (Table 1). DEM rapidly depleted pulmonary glutathione, a fall of 90% being noted over the first 2 h after administration of the drug. Lung glutathione concentrations rose significantly to beyond the normal levels associated with 1-day-old pups between 4 and 18 h after injection of DEM. By 48 h pulmonary glutathione concentrations had reverted to control values. A similar time course was observed for hepatic glutathione depletion by DEM (data not shown).

In preterm pups the age-related changes in pulmonary glutathione concentrations were more marked than in term pups (Table 2). Between 0 and 24 h after delivery, glutathione was depleted by 53% in control animals, relative to concentrations at birth. By 48 h, however, lung glutathione concentrations were significantly higher than at birth ($P < 0·05$). The administration of DEM depleted pulmonary glutathione by approximately 68% over a 2-h period. Twenty-four hours after the administration of DEM, lung glutathione levels were 137% above those of appropriate control pups. Between 24 and 48 h however, the normal increase (231%) in lung glutathione, observed in controls, did not occur in DEM-treated pups such that the final

Table 1. Glutathione depletion by DEM in term guinea-pig neonates.

Time after injection (h)	Lung glutathione (μmol (g tissue)$^{-1}$)	
	DEM	Oil
0	1·86±0·18	1·86±0·18
0·5	0·37±0·04†	ND
2	0·18±0·03†	ND
4	0·65±0·02†	ND
18	1·88±0·08†	ND
24	1·69±0·23†	1·17±0·11*
48	1·83±0·08	1·63±0·21

Term guinea-pig pups were administered DEM (100 mg kg^{-1}) in arachis oil or vehicle in a volume of 0·2 mL, (i.p.), 4–6 h post-partum. Values shown are means±s.e.m. *$P < 0·05$ compared with values at 0 and 48 h. †$P < 0·05$ compared with equivalent DEM-treated animals. ND, not determined.

Table 2. Glutathione depletion by DEM in preterm guinea-pig neonates.

Time after injection (h)	Lung glutathione (μmol (g tissue)$^{-1}$)	
	DEM	Oil
0	0·63±0·14	0·63±0·14
2	0·20±0·02†	ND
24	0·69±0·09†	0·29±0·06*
48	0·52±0·11†	0·96±0·06

Preterm guinea-pig pups were administered DEM (100 mg kg^{-1}) in arachis oil or vehicle in a volume of 0·2 mL, (i.p.), 30 min post-partum. Values shown are means±s.e.m. *$P < 0·05$ compared with values at 0 and 48 h. †$P < 0·05$ compared with equivalent DEM-treated animals. ND, not determined.

Table 3. The effects of oxygen exposure and DEM treatment on survival of term and preterm guinea-pigs.

	Survival (%)		
Treatment	0 h	24 h	48 h
Term			
Oil/air	100	100	89[a]
DEM/air	100	100	100[a]
Oil/O_2	100	100	100[a]
DEM/O_2	100	92	69[b]
Preterm			
Oil/air	100	100	100[a]
DEM/air	100	100	70[a]
Oil/O_2	100	90	80[a]
DEM/O_2	100	71	0[b]

Animals were administered arachis oil or DEM as described in the text (n=6–13 animals per group). Figures indicate % survival of animals following exposure to 21 or 95% oxygen for 48 h. Different superscripts (a, b) indicate statistically significant differences between 48 h survival curves. Statistical analysis does not include comparison of term and preterm animals.

glutathione level in the treated group was approximately one-half that of controls, and similar to the concentrations observed at birth.

Survival of pups exposed to hyperoxic conditions following DEM treatment

The survival of term and preterm guinea-pigs in 21% oxygen was not compromised by DEM treatment (Table 3). Over the 48-h period of the study, exposure to 95% oxygen did not cause any deaths in full-term, oil-injected controls. Exposure of DEM-treated term neonatal animals to 95% oxygen significantly increased mortality rates, a total of 4 out of 13 pups dying between 7 and 48 h after the start of treatment. Post-mortem examination revealed focal pulmonary haemorrhage and evidence of oedema, suggesting lung injury as the primary cause of death. One control (oil/air) pup died of unknown causes. Among preterm pups significant increases in mortality rates were associated only with DEM treatment of 95% oxygen-exposed pups (Table 3). In this group all animals died within 48 h. All deaths in this group were attributable to lung damage, as judged by pulmonary oedema and haemorrhage. Treatment with DEM, or hyperoxic exposure alone, was associated with 30 or 20% mortality rates, respectively. The survival curves of these groups did not significantly differ from that of controls.

Long-term effects of oxygen and DEM on tissue glutathione

At the end of the 48-h study period, lung wet weights were found to be unaltered by treatment with DEM, arachis oil, or exposure to oxygen, in either term or preterm animals (term pups: oil/air, 2·07±0·12 g; DEM/air, 2·24±0·13 g; oil/O_2, 2·40±0·13 g; DEM/O_2, 2·61±0·18 g, preterm pups: oil/air, 2·86±0·12 g; DEM/air, 2·98±0·33 g; oil/O_2, 2·86±0·12 g). Glutathione concentrations in the lungs, liver and BALF of term or preterm pups were unaltered by either exposure to 95% oxygen or treatment with DEM (Table 4). Glutathione concentrations in the lungs of preterm pups were significantly lower than in term pups ($P<0·001$). Oxidized glutathione levels accounted for less than 5% of the total glutathione measured, and were frequently not detectable (data not shown).

Table 4. Glutathione concentrations in lung, liver and bronchoalveolar lavage fluid (BALF) following 48-h oxygen exposure and diethylmaleate treatment.

	Glutathione		
Treatment	Lung (μmol (g tissue)$^{-1}$)	Liver (μmol (g tissue)$^{-1}$)	BALF (μM)
Term			
Oil/air	0·76±0·15	3·54±0·62	5·72±3·65
DEM/air	0·95±0·08	3·87±0·82	4·77±1·90
Oil/O_2	0·98±0·06	4·07±0·52	3·77±2·29
DEM/O_2	0·93±0·05	4·49±0·63	4·81±1·64
Preterm			
Oil/air	0·59±0·11	2·87±0·39	8·87±2·07
DEM/air	0·52±0·11	3·02±0·33	23·39±9·67
Oil/O_2	0·53±0·09	3·66±0·49	11·31±2·71
DEM/O_2	ND	ND	ND

Animals were administered arachis oil or DEM as described in the text (n=6–13 animals per group). Figures are means±s.e.m. ND, not determined.

Table 5. The effects of DEM treatment and 48-h oxygen exposure on bronchoalveolar lavage fluid (BALF), protein concentration and leucocyte numbers.

			Leucocytes × 10^4	
Treatment	BALF (mL)	Protein (mg mL^{-1})	Total cells	Neutrophils
Term				
Oil/air	8·0±0·3[a]	0·23±0·05[a]	3·00±0·5[a]	0·21±0·07[a]
DEM/air	8·1±0·2[a]	0·31±0·05[a]	6·00±1·4[b]	0·12±0·07[a]
Oil/O_2	8·4±0·2[a]	0·28±0·03[a]	4·90±0·5[a]	0·94±0·33[a]
DEM/O_2	7·9±0·4[a]	0·69±0·25[a]	6·60±0·3[b]	3·40±1·93[b]
Preterm				
Oil/air	8·4±0·2[a]	0·34±0·11[a]	2·70±0·6[a]	0·33±0·10[a]
DEM/air	7·9±0·3[a]	0·24±0·05[a]	2·50±0·2[a]	0·18±0·04[a]
Oil/O_2	7·7±0·5[a]	0·38±0·04[a]	3·50±0·6[a]	1·26±0·70[b]
DEM/O_2	ND	ND	ND	ND

Animals were administered arachis oil or DEM as described in the text (n=6–13 animals per group). Figures are means±s.e.m. Different superscripts (a, b) indicate statistically significant differences between groups. Statistical analysis does not include comparison of term and preterm animals. ND, not determined. Analysis of variance. Term animals: effect of DEM noted on BALF total cells $P<0·005$, F=9·64 (1,24); effect of O_2 noted on BALF neutrophils $P<0·05$, F=6·48 (1,22); effect of DEM noted on BALF protein $P<0·05$, F=6·32 (1,33); effect of O_2 noted on BALF protein $P<0·05$, F=8·20 (1,33). Preterm animals: effect of O_2 noted on BALF neutrophils $P<0·05$, F=6·42 (1,33).

Effects of DEM and hyperoxia on lung injury

BALF recovery was not significantly altered by any of the treatments (Table 5). Exposure of term pups to 95% oxygen for 48 h elicited none of the changes in BALF characteristics normally associated with lung injury. Microvascular permeability, as indicated by BALF protein concentrations, was normal, and no influx of inflammatory cells was observed. Treatment of 21% oxygen-exposed pups with DEM similarly had no effect on BALF protein concentrations or neutrophil numbers, but did significantly increase total leucocyte numbers. The combination of DEM administration and exposure to 95% oxygen significantly elevated BALF protein concentrations (3-fold), BALF total leucocyte numbers (2-fold) and BALF neutrophil numbers (16-fold).

In preterm pups exposed to 21% oxygen, treatment with DEM had no effect on BALF protein concentrations. No measurements were possible in 95% oxygen-exposed pups treated with DEM, as no animals survived this protocol. Oxygen exposure elicited an increase in BALF neutrophil numbers, with no changes in BALF total leucocyte numbers, or BALF protein concentrations.

Discussion

The administration of DEM to rodents transiently depletes glutathione through conjugation catalysed by the glutathione-*S*-transferases (Plummer et al 1981). DEM is not a specific glutathione-depleting agent and has a number of other effects (Meister 1991), including general depletion of intracellular thiols, renal damage and lipid peroxidation. The low doses of DEM employed (12% of those generally applied in rats) in the study may have avoided some of these confounding factors, and indeed the lack of significant DEM-induced increases in mortality rates or tissue injury in 21% oxygen-exposed animals, supports this assertion. All deaths among DEM-treated, 95% oxygen-exposed pups could be directly attributable to lung injury. Indirect lung injury has not been demonstrated to be a problem associated with the use of DEM. In the present study, a single dose of DEM administered to term or preterm guinea-pig neonates, significantly depleted pulmonary glutathione concentrations over a period from 30 min to 4 h. As has been previously reported in studies using adult rats (Deneke et al 1985b; Weber et al 1990), the subsequent recovery of glutathione levels led to an overshoot of normal control values. This overshoot was of greatest magnitude in preterm pups at 24 h. Treatment of preterm pups with DEM appeared, however, to disrupt the normal developmental changes in glutathione levels, such that by 48 h, control concentrations had not been attained. Following DEM administration, levels of pulmonary glutathione fell to similar levels in both term and preterm animals. As DEM does not deplete the mitochondrial pool (Plummer et al 1981) it can be inferred that any difference in tissue glutathione between term and preterm pups is present in the cytosol. This cytosolic pool of glutathione would ultimately appear to underly the observed differences in susceptibility to lethal hyperoxia-induced lung injury.

Guinea-pig neonates exposed to normoxic or hyperoxic conditions for 48 h were administered two doses of DEM on the first day of the study, 6 h apart. On the basis of the time-course study it was assumed that pulmonary glutathione concentrations were depleted for no more than 12–18 h by this approach. The depletion protocol employed was selected to be transient and covered only the first 25% of the exposure to high oxygen tension. Indeed, given the overshoot of glutathione on recovery, in preterm pups, pulmonary concentrations may have been actually elevated in DEM-treated animals for a significant proportion of the study period.

Administration of DEM to guinea-pig neonates did not appear to have any toxic effects, although a significantly lower dose than that used in other studies (Deneke et al 1985b; Weber et al 1990) had to be employed, as preterm animals suffered hepatic injury at doses previously employed with adult rats (0·8 g kg^{-1}). Exposure of term and preterm pups to 95% oxygen for 48 h has been shown to be associated with 10 and 15% mortality, respectively (Kelly et al 1991). In the present study, rates of mortality observed in oxygen-exposed animals were consistent with these previous findings, preterm pups being more susceptible to the lethal effects of oxygen than term animals. Transient depletion of pulmonary glutathione significantly increased levels of mortality, an effect particularly noticeable in the preterm group, none of which survived the protocol. All deaths of DEM 95% oxygen-treated animals were associated with evidence of pulmonary damage, but it may be hypothesized that damage to other organs may have also contributed to the death of the animals. Chronic depletion of glutathione using buthionine sulphoximine is associated with injury to lung, brain, intestine and eyes (Martensson et al 1989a, b, 1990; Jain et al 1991; Meister 1991), which may be exacerbated in hyperoxic conditions. These buthionine sulphoxide-induced tissue injuries would appear to be related to depletion of the mitochondrial pool (Meister 1991), leading to mitochondrial degeneration and loss of function. In the present study the mitochondrial pool was unlikely to have been affected by the transient depletion regime. The increases in mortality are consistent with the findings of Deneke et al (1985b) who demonstrated that transient depletion of lung glutathione with DEM accelerated oxygen-induced death in adult rats. Similarly, Weber et al (1990) demonstrated acceleration of the onset of seizures and death in hyperbaric oxygen.

In addition to an acceleration of oxygen-induced death, particularly in preterm pups, DEM was found to hasten the onset of lung injury. After 48 h of 95% oxygen-exposure, the level of injury sustained was normally below detectable levels, using the available indices. In oil-treated control term pups exposed to 95% oxygen, no significant increases in BALF protein concentrations or inflammatory cell numbers were observed. Preterm pups, which are more susceptible to oxidative lung injury than term pups (Kelly et al 1991), were found to have increased neutrophil numbers in the lungs after 48-h oxygen exposure. The magnitude of this neutrophil influx was, however, approximately 60% of that previously observed at 72 h (Langley & Kelly 1992), when BALF protein concentrations were also increased. Oxygen-exposed term pups treated with DEM, unlike the oil controls, showed evidence of increased pulmonary microvascular permeability. Increased capillary permeability leads to pulmonary oedema and eventually hypoxia and death (Massaro 1986). Glutathione-depleted, oxygen-exposed term neonates were found to have 50% more neutrophils in their lungs than preterm pups exposed to oxygen for 72 h. This is further consistent with the hypothesis that DEM accelerates the development of oxidative lung injury.

From the data presented, it can be postulated that in the newborn, glutathione is an essential component of the antioxidant array required to protect the lungs from oxidative damage. Whilst the importance of glutathione as an antioxidant has been previously demonstrated, the present study is novel in its observations of premature neonates. In this respect glutathione may function either as a direct scavenger of free radicals or as the substrate for glutathione peroxidase. Over the first 48 h of life, preterm pups were found to have significantly less pulmonary glutathione than their full-time counterparts. This difference was most marked at 24 h post-partum, at which time pulmonary

glutathione concentrations of preterm animals were barely above those of term animals shortly after DEM administration. The preterm guinea-pig is more susceptible to oxidative injury than term pups (Kelly et al 1991), and pulmonary glutathione deficiency may, coupled with deficiencies of antioxidant enzymes, explain this reduced tolerance of hyperoxia. The 100% mortality rate among DEM-treated oxygen-exposed preterm pups demonstrates the essential nature of glutathione in preterm pups undergoing oxygen treatment. This finding will have to be considered in the development of strategies for glutathione therapy, currently being considered for the management of preterm infants.

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