Protection by N-acetylcysteine against pulmonary endothelial cell damage induced by oxidant injury

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ABSTRACT: The protective effect of N-acetylcysteine (NAC) against oxidant lung injury was investigated in a model of acute immunological alveolitis in the rat. Intraluminal immune complex deposition into rat lungs, induced by intratracheal infusion of immunoglobulin G (IgG) anti-bovine serum albumin (BSA) antibodies and intravenous injection of the antigen, caused lung damage associated with a marked decrease in [14C]-5-hydroxytryptamine ([14C]5HT) uptake capacity, taken as a biochemical marker of endothelial cell function.

The oral administration of a single dose of NAC (2 mmol·kg⁻¹) 60 min before antigen/antibody (Ag/Ab) treatment was effective in preventing pulmonary endothelial cell [14C]5HT uptake loss induced by immune complex deposition. The mechanisms involved in this lung protective action of NAC were investigated by studying the antioxidant activity of NAC on hypoxanthine/xanthine oxidase-induced lung damage in vitro, and the effectiveness of the drug as lung glutathione (reduced form) (GSH) precursor in diethylmaleate-depleted rats.

The results obtained provide further evidence on the ability of NAC to reduce the susceptibility of lung tissue to free radical-induced damage, by potentiating the antioxidant defence systems.

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It is widely held, that oxygen free radicals (OFR) play an important role in the pathogenesis of many inflammatory diseases [1-3]. Although OFR are normally generated by inflammatory cells as a defence mechanism against infectious agents, such as viruses and bacteria, the release of large amounts of these oxygen metabolites, exceeding the antioxidant capacities of the tissues, may result in cell damage. A great deal of information has been accumulated about the role of OFR in oxidant lung injury [4-6]. The pathogenesis of the lung damage occurring during some acute and chronic diseases, such as adult respiratory distress syndrome (ARDS) and emphysema, has been related to the harmful action of OFR generated by the nicotinamide adenine dinucleotide phosphate reduced form (NADPH)-oxidase metabolic pathway of activated phagocytic cells, mainly neutrophils and macrophages [7-9]. Since protection against oxidant injury can be afforded by treatments preventing OFR generation or accumulation, or increasing the antioxidant defence mechanisms of the lung, the availability of drugs with OFR scavenging activity may be relevant for the therapy of many pulmonary disease states.

In recent years, increasing interest has arisen in N-acetylcysteine (NAC) as a potential therapeutic agent in oxidant-mediated disorders [10-12]. This compound, which has been widely used as a mucolytic drug for the treatment of chronic obstructive lung diseases [13-15], is a potent antioxidant agent, showing protective effects in several models of lung injury [16, 17]. The antioxidant activity of NAC appears to be related to at least two mechanisms, i.e. the nucleophilic properties of the molecule accounting for its OFR scavenger activity and the capacity to support glutathione (reduced form) (GSH) synthesis [18-20].

Lung endothelial cells are a primary target of injury during pulmonary diseases in which OFR are involved. It has been demonstrated that serotonin (5HT) uptake is an active process representing a specific endothelial cell function, showing marked sensitivity to extracellular generated OFR [21]. In the present paper, 5HT uptake was taken as a specific marker of pulmonary endothelial cell function, in order to investigate the protective effects of NAC against oxidant lung injury occurring during acute immunological alveolitis in rats. NAC, orally administered as a single dose, was effective in preventing lung endothelial cell damage induced by intrapulmonary immune complex deposition. As shown by the results obtained in the experiments carried out in the isolated perfused rat lung and in GSH-depleted rats, the mechanism involved in such a protective effect can be related either to the OFR scavenger activity of the molecule or to the effectiveness of NAC in sustaining GSH synthesis.
Materials and methods

Animals

All of the experiments were performed on adult male Sprague-Dawley rats, weighing about 250 g (Charles River, Calco, Italy). The animals were maintained at 20–22°C, 60% relative humidity, with a 12 h light-dark cycle. They had free access to commercially available rodent pelleted diet and water, except those in fasting experiments described below.

Immune complex alveolitis

Rat immunoglobulin G (IgG) anti-bovine serum albumin (BSA) (Fluka Chemie, Switzerland) was obtained and partially purified as described previously [22]. Briefly, the IgG-enriched fraction was isolated from hyperimmune rat serum by precipitation with saturated ammonium sulfate (50%), followed by dialysis against 0.005 M phosphate-buffered saline (PBS) medium (Seromed, Germany) and diethyl aminoethylcellulose ion exchange chromatography.

Immune complex alveolitis was induced according to Johnson and Ward [23]. The animals were divided into the following groups: untreated rats, negative controls (IgG anti-BSA, intratracheal instillation (i.t.)), positive controls (IgG anti-BSA i.t. + BSA, intravenous injection (i.v.)) and positive controls + 2 mmol·kg⁻¹ NAC, oral administration (o.e.). Rats were anaesthetized with ether and 0.25 mg of IgG anti-BSA antibodies were administered by slow intratracheal instillation via a polyethylene catheter (1 mm calibre) passed through a needle inserted into the trachea. The antigen BSA was immediately administered intravenously at the dose of 10 mg in 0.5 ml 0.9% NaCl. NAC was orally administered at 1 ml 0.9% NaCl·100 g⁻¹ body weight, 30 min before IgG anti-BSA/BSA treatments. After instillation, the catheter and needle were removed from the trachea, and the incision sutured. Five hours later the lungs were removed and isolated as described below for ¹⁴C-labelled serotonin ([¹⁴C]5HT) uptake.

Isolated perfused lung preparation

Isolated lung preparation was performed according to the method described by Chiang and Vorlkel [24], with minor modifications. The animals were injected with pentobarbital (50 mg·kg⁻¹). After tracheal exposure, a cannula was inserted and ventilation started with room air at 50 breath·min⁻¹, at a volume of approximately 1 ml·100 g⁻¹ body weight. After opening the chest, an intracardiac injection of 100 U of heparin was given, and a cannula inserted into the pulmonary artery and secured. The left side of the heart was removed and perfusion begun through the pulmonary artery cannula at a slow rate, using a Kreb’s-Henseleit buffer containing 4% Ficoll 70. The lungs were then removed, placed in a warmed humidified chamber and a positive expiratory pressure of 2.5 cmH₂O was imposed. The perfusion was carried out in a non-recirculating manner, at a final rate of 4 ml·100 g⁻¹ body weight·min⁻¹. The lungs were perfused with 30 ml perfusate to wash the pulmonary circulation free of blood. Pulmonary artery mean pressure and airway pressure were continuously monitored using a Statham P102 pressure transducer (Gould Electronics, Cleveland, USA) connected to a signal amplifier and to a recorder (Gould Electronics, Cleveland, USA). A stable baseline was obtained after 15 min perfusion.

Pulmonary [¹⁴C]5HT uptake

The pulmonary clearance of 5HT, taken as a biochemical index of pulmonary endothelial cell function, was evaluated as described previously by Steenberg et al. [25]. Isolated lungs were perfused with Kreb’s-Henseleit buffer containing [¹⁴C]5HT (spec. activity 57 mCi·mmol⁻¹, Amersham Int., UK) at the concentration of 80,000 cpm·ml⁻¹. Iproniazid (Sigma, USA) was included in the perfusion medium, at the concentration of 10⁻⁴ M, in order to block the endothelial catabolism of serotonin [21]. The effluent from the pulmonary vein was collected and the radioactivity present in 1 ml aliquots was measured by scintillation spectrometry, in 10 ml Lumagel scintillation cocktail (LUMAC) in a TRI-CARB 4530 spectrometer (Packard), with an efficiency of about 90%. Pulmonary serotonin uptake was calculated as the arterovenous difference in [¹⁴C]5HT concentration.

Hypoxanthine/xanthine oxidase perfusion

Oxidant lung injury was induced in vitro by perfusion with hypoxanthine/xanthine oxidase, one of the most used OFR generating systems [26]. Lungs were perfused with 1 mM hypoxanthine (HYP) (Boehringer Mannheim) and 40 μM-xanthine oxidase (XO) (Boehringer Mannheim, spec. activity 1 U·mg⁻¹) in 50 ml of Kreb’s-Henseleit buffer, by using a recirculating perfusion system. Oxidant injury was estimated by measuring [¹⁴C]5HT uptake, as described above, before and after fixed time intervals of perfusion with HYP/XO.

The effects of NAC on HYP/XO-induced [¹⁴C]5HT uptake decrease was investigated by including the drug at the concentration of 1 mM in the perfusion medium, 1 min before HYP/XO addition. The involvement of OFR in HYP/XO-induced lung damage was checked, evaluating the protective effects of a perfusion with 10 U·ml⁻¹ superoxide dismutase (SOD) (5,000 U·mg⁻¹, Boehringer Mannheim) and 15 μg·ml⁻¹ catalase (CAT) (65,000 U·mg⁻¹, Boehringer Mannheim) and the reduction of cytochrome C (0.57 mg·ml⁻¹, Sigma, USA) in the perfusate [25].

Lung GSH depletion and measurement

GSH depletion was induced in overnight fasted rats by an intraperitoneal injection of diethylmaleate (DEM) (Fluka...
Chemic, Switzerland), at the dose of 150 mg·kg⁻¹ in 1 ml·kg⁻¹ body weight propylene glycol.

The effects of the oral administration of NAC on lung GSH levels in DEM-treated rats were investigated in dose-response and time-course experiments. In both studies, DEM was administered 30 min before NAC treatment. In dose-response experiments, NAC was administered at the doses of 0.5, 1 and 2 mmol·kg⁻¹ and rats were sacrificed 240 min after the treatment.

In time course studies, rats were sacrificed 30, 120 and 240 min after a single administration of 2 mmol·kg⁻¹ NAC. After decapitation, lungs were rapidly removed and immediately frozen in a dry ice-ethanol mixture. Lungs were then homogenized with an ultra-turrax homogenizer (Ika Werk, Germany), in 2 ml of 5% 5-sulphosalicylic acid, and centrifuged at 45,000 × g, 4°C for 20 min. The supernatants were used for the spectrophotometric determination of total GSH, by using the enzymatic recycling assay based on GSH-reductase (120 U·mg⁻¹, Boehringer Mannheim) described by Anractep (27). Sample GSH concentrations were calculated from a standard curve of GSH (Boehringer Mannheim).

Statistical evaluation

Data are expressed as mean±SEM of six animals (alveolitis and GSH studies) and five individual lungs (HYP/XO studies). Significance of the differences between groups was determined by unpaired Student's t-test. A value of p<0.05 was regarded as significant. In GSH time-course studies, the statistical evaluation was performed by using the analysis of variance with the factorial design (SAS rel. 6.06 PROC GLM with contrast option). A value of p<0.05 was regarded as significant.

Results

As reported in the methods, lung injury was quantified as loss of pulmonary [³¹C]5HT uptake, which was taken as an index of pulmonary endothelial cell function. As shown in figure 1, when lungs from rats which received an intratracheal instillation of IgG anti-BSA antibodies and an intravenous injection of the antigen (BSA) were isolated and perfused with [³¹C]5HT, a marked decrease in the uptake of the amine was observed. As clearly shown, [³¹C]5HT uptake was significantly reduced in positive control animals 5 h after antigen/antibody (Ag/Ab) treatments (mean±SEM 1,816±22 pmol·min⁻¹·g⁻¹ tissue) when compared to uptake values observed in untreated and negative control rats (2,150±73 and 2,143±40 pmol·min⁻¹·g⁻¹ tissue, respectively). The difference was statistically significant (p<0.01, unpaired Student's t-test).

On the basis of these observations, the experiments carried out in order to investigate the protective effect of NAC against immune complex deposition-induced lung injury were performed as follows. NAC was orally administered to rats, at the dose of 0.5 and 2 mmol·kg⁻¹, 30 min before IgG anti-BSA i.t./BSA i.v. treatments. Lungs were removed 5 h after NAC administration and perfused for [³¹C]5HT uptake measurements.

The results obtained in these experiments are illustrated in figure 2. As can be observed, the oral administration of NAC at the dose of 2 mmol·kg⁻¹ was effective in preventing [³¹C]5HT pulmonary uptake loss induced by intrapulmonary immune complex deposition. Uptake values were 1,816±22 pmol·min⁻¹·g⁻¹ tissue in lungs from vehicle-treated rats (group B) and 1,969±40 pmol·min⁻¹·g⁻¹ tissue in lungs from NAC-treated rats (group D). The difference was statistically significant (p<0.01, unpaired Student's t-test). No significant protective effects were observed at the lower dose of 0.5 mmol·kg⁻¹.

In order to investigate the mechanisms involved in this protective effect of NAC against lung injury, the ability of NAC to prevent OFR-induced pulmonary damage in the isolated rat lung was estimated. OFR were generated by HYP/XO lung perfusion and, in this case also, pulmonary damage was estimated as [³¹C]5HT uptake loss.

When isolated rat lungs were perfused with HYP/XO system, a time-dependent reduction in pulmonary [³¹C]5HT uptake was observed. As shown in figure 3, about 80% of circulating [³¹C]5HT was removed from the pulmonary circulation during perfusion with HYP alone at each time indicated. On the other hand, during HYP/XO perfusion a progressive decrease in [³¹C]5HT uptake did occur, resulting in about 50% reduction of the uptake after 20 min perfusion.

The involvement of OFR in HYP/XO-induced [³¹C]5HT uptake loss was demonstrated by the protective effects produced by the presence of the enzymatic scavengers, superoxide dismutase (SOD) and catalase (CAT), in the perfusion medium. As illustrated in figure 3, these enzymes almost completely prevented the reduction of the uptake induced by HYP/XO.
Fig. 2. – Protective effect of the oral administration of N-acetylcysteine (NAC) against intrapulmonary immune complex-induced ³⁴C-hydroxytryptamine ([³⁴C]5HT) uptake loss in rat lung (n=6). Group A: negative controls. Group B: positive controls. Group C: positive controls + NAC 0.5 mmol·kg⁻¹ os: oral administration. Group D: positive controls + NAC 2 mmol·kg⁻¹ os: oral administration. *: p<0.01 as compared to group B. Note that vertical axis does not start at zero.

Fig. 3. – Time-course of HYP/XO-induced [³⁴C]5HT uptake loss in the isolated perfused rat lung. x: HYP; O: HYP/XO; •: HYP/XO+SOD 100 U·ml⁻¹+CAT 600 U·ml⁻¹. Each point is the mean±SEM of six determinations. *: p<0.01 as compared to HYP/XO 20 min. HYP: hypoxanthine; XO: xanthine oxidase; [³⁴C]5HT: ³⁴C-hydroxytryptamine; SOD: superoxide dismutase; CAT: catalase.

The protective effect of NAC against HYP/XO-induced pulmonary [³⁴C]5HT uptake loss is illustrated in figure 4. In these experiments, [³⁴C]5HT uptake was measured before and after 20 min perfusion with HYP/XO, in the absence (control lungs) and in the presence of 1 mM NAC. As shown in panel A, a marked reduction of [³⁴C]5HT uptake was observed in control lungs. Mean±SEM values from five individual lungs were 3,045±306 and 1,915±422 pmol·min⁻¹·g⁻¹ tissue, before and after HYP/XO perfusion, respectively. Pulmonary uptake loss was 1,130±184 pmol·min⁻¹·g⁻¹ tissue, corresponding to a 37% decrease from basal value.

When 1 mM NAC was included in the perfusion medium, a clear decrease in [³⁴C]5HT uptake loss was observed (fig. 4). Mean uptake values for NAC-treated lungs were 2,748±147 and 2,232±119 pmol·min⁻¹·g⁻¹ tissue before and after 20 min HYP/XO perfusion, respectively. The uptake loss was 514.2±62 pmol·min⁻¹·g⁻¹ tissue, corresponding to an 18.7% decrease from basal value. This preventive effect of NAC against HYP/XO-induced [³⁴C]5HT uptake loss was statistically significant (p<0.01, Student's unpaired t-test).

As a further antioxidant mechanism of action against oxidant lung injury, the ability of NAC to sustain pulmonary GSH synthesis in vivo was investigated. As described in the methods, lung GSH depletion was induced by an intraperitoneal treatment with DEM (150 mg·kg⁻¹) and the ability of NAC to restore lung GSH content was studied in dose-response and time-course experiments. In figure 5 the effects of the oral administration of different doses of NAC on pulmonary GSH levels in DEM-treated rats are illustrated. GSH concentrations were measured 240 min after NAC treatment.
Effects of the oral administration of NAC on lung GSH levels from DEM-treated rats (n=6). GSH concentrations were measured 240 min after NAC treatment. *: p<0.05; **: p<0.01 as compared to DEM. NAC: N-acetylcysteine; GSH: glutathione (reduced form); DEM: diethylmaleate.

As can be observed, NAC was effective in restoring GSH levels in a dose-dependent manner in the dose range 0.5–2 mmol·kg⁻¹.

The results obtained in time-course experiments are illustrated in figure 6. As can be seen, GSH concentration was reduced to 23% of the basal value 30 min after DEM administration. Afterwards, lung GSH levels partially recovered between 1 and 4.5 h from DEM treatment to 45% of the basal levels. The oral administration of 2 mmol·kg⁻¹ NAC 30 min after DEM treatment was effective in increasing the rate of GSH resynthesis, inducing the recovery of basal GSH values within 240 min (p<0.001, analysis of variance (ANOVA) test with contrast option).

Discussion

OFR-mediated tissue injury has been suggested to be involved in numerous pathological processes. In particular, a vast amount of international literature has demonstrated that oxidant lung injury plays a major role in the pathogenesis of many pulmonary disease states, such as ARDS and emphysema. Considerable evidence supports the concept that infiltrated neutrophils play a central role in the process of injury, as a consequence of OFR generation [5, 9, 28]. Activated neutrophils, in fact, adhere to the endothelium and cause microvascular endothelial cell injury by releasing toxic oxygen metabolites, resulting in microvascular leakage and pulmonary oedema. Moreover, increased production of OFR may occur within endothelial cells [29], probably involving xanthine/xanthine oxidase reactions [30], causing decrease of intracellular antioxidant systems such as glutathione.

Hence, compounds with free radical scavenger properties, which can easily penetrate into the cells increasing the intracellular antioxidant content, should represent a valid pharmacological intervention against OFR-induced injury to endothelium. Among the best known antioxidant agents, NAC represents one of the most wide-ranging and interesting drugs because of its double mechanism of action: the first at an endocellular level, as a precursor in the synthesis of the physiological antioxidant GSH; the second, at an extracellular level, by acting directly with OFR because of NAC nucleophilic properties.

In the present study, the protective effects of NAC against oxidant lung injury were investigated using a model of experimentally-induced immunological alveolitis in the rat. Lung injury was induced by IgG-containing immune complex deposition into the lungs, resulting in complement activation and recruitment of large numbers of neutrophils in the interstitial compartment of the lung and into the alveolar spaces [31]. Damage to the endothelial cells and macroscopically evident haemorrhages occur in the lung as a result of neutrophil granulocyte activation, adhesion to endothelial cells and OFR release [32]. In fact, a great deal of evidence has been produced indicating that IgG immune complex-induced lung injury is OFR-dependent. For example, the involvement of hydroxyl radical as a main cause of tissue injury in this model has been strongly suggested by the effectiveness of intra-tracheally administered catalase to suppress the development of immune complex alveolitis [33]. An iron-dependent hydroxyl radical formation appears to be involved, as treatment with iron chelating agents, such as deferoxamine, is markedly protective [34]. Again, the administration of superoxide dismutase is partially protective, probably during the early development of injury, where superoxide anion generation seems to play a key role in the initiation of the injury [35]. Moreover, xanthine oxidase inhibitors, such as allopurinol, are effective in suppressing pulmonary immune complex vasculitis after systemic treatment, indicating a role for this O₂−-generating enzymatic system in oxidant lung injury [32].

Using [³⁵S]5HT uptake as a specific index of pulmonary endothelial cell function [25], we have demonstrated that when lungs from rats with experimentally-induced alveolitis were isolated and perfused with [³⁵S]5HT, a dramatic decrease of the uptake capacity does occur. Such an impairment of endothelial cell function was valuable 4 h after Ag/Ab treatment and reflects
Oxygen

Oral

Oral

subjects [19] and in patients with chronic obstructive

convincing evidence about the relevance of the antioxidant

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in this model NAC exercises a protective effect, reducing

metabolites generated by polymorphonuclear neutrophils:

studying the pulmonary damage induced by reactive

may represent a more relevant mechanism of lung protec­

GSH synthesis promotion. As demonstrated

OFR, as reported by their expert technical assistance.

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acetylcysteine and exacerbation rates in patients with chronic

extensive cellular injury to membrane receptors, metabolic
pumps and energy sources available for transport [36]. This
detrimental effect on endothelial cell activity, induced by in
vivo intrapulmonary immune complex de­
position, was counteracted by the oral administration of a
single dose of NAC (2 mmol·kg–1) 30 min before Ag/Ab treatment.

Protection against immune complex-induced endothelial cell
damage by NAC can be related to the ability of the
compound to inactivate OFR, either directly or through
GH synthesis promotion. It has been shown that NAC
is a powerful scavenger of reactive oxygen species, such as
hydroxyl radical and the myeloperoxidase-derived
oxidant hypochlorous acid, either in cell free systems or
in isolated cell models [18]. NAC, as well as GSH, in­
teracts with superoxide anion formed by the xanthine/
xanthine oxidase system and with hydrogen peroxide with
formation of the NAC disulphide [19]. We have also
found, like other authors [18], that NAC is able to sup­
press oxidants produced by stimulated phagocytes (unpublished
data).

The results obtained in this study, concerning the abili­
ty of NAC to prevent lung endothelial cell [35]SHT
uptake loss induced by HYP/XO perfusion in vitro, rep­
resent a further indication of the involvement of OFR
scavenger actions of NAC in lung protection. The
relevance of such a mechanism in endothelium protec­tion
by NAC has recently been demonstrated by COTGREAVE
et al. [35], who indicated the significance of a direct
scavenging effect of NAC in human endothelium cyto­
protection in addition to GSH synthesis stimulation within
endothelial cells.

On the other hand, the effectiveness of NAC to sup­
port GSH synthesis in the lung in vivo, as demonstrated by
the results obtained in DEM-treated rat experiments,
can represent a more relevant mechanism of lung protec­tion
by NAC when orally administered. Indeed, oral
administration with the dose of NAC, found to exert a pro­
tective effect against immune complex-induced lung in­
jury, was very effective in replenishing GSH lung content.

The model of acute pulmonary alveolitis in rats has been
proposed as one of the most suitable models for
studying the pulmonary damage induced by reactive
metabolites generated by polymorphonuclear neutrophils:
in this model NAC exercises a protective effect, reducing the
susceptibility of the pulmonary tissue to damage
caused by free radicals, by potentiating the antioxidant
defence mechanisms of the lung. Protective effects of
NAC were also reported by BERNARD et al. [16] and
WEiJER et al. [37] in two other experimental models of
ARDS in animals, where NAC was found effective in
preventing lung damages occurring during endotoxin in­
fusion in sheep and pulmonary microembolism in rats.

In addition to the very well-known antidotal effective­
ness of NAC against drug-induced hepatotoxicity [10],
convincing evidence about the relevance of the antioxidant
properties of this compound in pneumoprotection has also
been produced in man. It has recently been found, that
NAC orally administered is able to increase GSH levels
in plasma and in bronchoalveolar lavage fluid in normal
subjects [19] and in patients with chronic obstructive
pulmonary disease (COPD) [38]. Moreover, similar in­
creases in GSH concentrations were observed in plasma
and red cells from ARDS patients orally treated with
NAC, together with an improvement of cardiopulmonary
physiology [39].

In conclusion, the results obtained in this study, on the
effects of NAC in the model of acute immunological
alveolitis, provide further evidence on the protective ac­
tion of NAC as an OFR scavenger and GSH synthesis
promoter at pulmonary level, giving more indications about
the potential prophylactic use of NAC in patients
with inflammatory lung diseases where an oxidant­
antioxidant imbalance plays a major role, such as emphy­
sema, ARDS and COPD.

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