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G.L. Plaa, P. du Souich, S. Erill, editors.

MOLECULAR SCAVENGING - NEW USE OF OLD DRUGS

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INTRODUCTION

There exist an extensive number of potentially reactive compounds of both exogenous and endogenous origin. These may be either arylating or oxidizing species and give rise to cytotoxicity and/or mutagenicity. The scavenging and detoxification of such reactive intermediates is generally catalyzed by endogenous defence systems such as glutathione linked processes, antioxidants such as vit E or the superoxide and hydrogen peroxide metabolizing enzymes, superoxide dismutase and catalase.

Examples of exogenous compounds which could serve as scavengers of reactive species are various antioxidants which interact with free radicals and prevent lipid peroxidation. There are also several examples of metal chelating agents. There are however not many drugs that are molecular scavengers. One example is the thiol reagent sodium mercaptoethane sulfonate (mesna, uronitexan) (1). This drug is used to prevent the urotoxic effects of oxazaphospharine cytostatics (cyclophosphamide, ifosfamide etc.). Mesna is concentrated in the kidney and urine and interacts with the toxic products formed from for instance cyclophosphamide, particularly acrolein. Another thiol containing drug which has been used for the same purpose is N-acetylcysteine (NAC). NAC is also used therapeutically in several more clinical disorders. It is for instance commonly used as a mucolytic in chronic obstructive pulmonary diseases beeing administred either topically or orally (2,3). It is also used as an antidot against paracetamol induced hepatotoxicity. It is then usually given in large i.v. doses. NAC does also seem to prevent renal stones in cystinuria.

The mucolytic efficacy of NAC applied directly to the airways be nebulization or direct instillation is well established. The proposed mechanism of the mucolytic action of NAC-administered by this route is by direct cleavage of disulphide bridges between glycopeptides, with a resultant decrease in the viscosity of bronchial secretion (4). However, there are also results indicating a mucolytic action of oral NAC treatment. Reductions of the frequency of the exacerbations in patients with chronic bronchitis on oral treatment with NAC have been reported (3).

Orally administred NAC does however not reach the airways in high enough concentrations in order to reduce disulphide bounds in the mucopolypeptides. Thus the mechanism of orally administred NAC is still unknown.

NAC is a thiol containing compound which gives it nucleophilic properties. NAC may thus interact and detoxify reactive electrophiles and free radicals either by conjugation or reduction. In many tissues and cells, NAC is also readily deacetylated to form cysteine, which efficiently supports glutathione (GSH) biosynthesis (5). In fact, the protection afforded by NAC against paracetamol-induced hepatotoxicity has been demonstrated to result primarily from stimulated GSH biosynthesis (6). GSH is a tripeptide which is present in high concentrations in most cells. GSH has many cellular functions, one of the most important beeing its ability to inactivate reactive compounds through conjugation and/or reduction (7). It is clear that there is an intimate link between cigarette smoking and the occurrence of lung diseases such as chronic bronchitis or emphysema. Thus NAC could serve as a protective agent towards reactive electrophiles and free radicals in cigarette smoke and/or towards active oxygen species and hypohalides formed during secondary inflammatory processes, either itself or by supporting GSH biosynthesis.

The present manuscript summarizes some of our findings regarding the interaction of NAC with various reactive species, the protective effect of NAC against their toxicity and the possible mechanisms by which this may occur.

RESULTS AND DISCUSSION

NAC may serve as a GSH precursor and this has been demonstrated to occur in cells isolated from various organs, in perfused organ such as the lung, liver, intestine and kidney as well as *in vivo* (6,8). Both NAC and GSH may interact with and inactivate most reactive electrophiles either by conjugation or reduction. Whereas the NAC dependent reactions are always nonenzymatic, GSH dependent conjugation of electrophiles and GSH dependent reduction of hydroperoxides are generally catalyzed by glutathione transferase and glutathione peroxidase, respectively. Thus, within the cell the GSH dependent inactivation reaction are greatly facilitated.

The protective effect of NAC against the toxicity of reactive electrophiles has been demonstrated using various chemicals and experimental systems. NAC has for instance been shown to protect isolated hepatocytes from acrolein induced toxicity (10). Acrolein is a reactive aldehyde which is a major component of cigarette smoke. Cigarette smoke contains many more reactive components including free radicals, as well as many components which are not reactive *per se* but are metabolized to reactive products.

We have studied the effect of cigarette smoke and cigarette smoke condensate in a number of *in vitro* systems such as human bronchial fibroblasts and epithelial cells, isolated lung cells from rat and the isolated perfused rat lung. Cigarette smoke and all of its major condensation fractions are extremely toxic in

TABLE I
MECHANISMS OF PROTECTIVE EFFECT OF NAC AGAINST DIFFERENT TYPES OF TOXIC CHEMICALS IN VARIOUS EXPERIMENTAL SYSTEMS

	Experimental systems	Mechanisms: Direct interaction	Mechanisms: Through stimulated GSH synthesis
Cigarette smoke and cigarette smoke condensate:	Human fibroblasts	+	
	Human epithelial cells	+	
	Isolated hepatocytes	+	
	Isolated perfused lung		+
Acrolein:	Human fibroblasts	+	
	Human epithelial cells	+	
	Isolated hepatocytes	+	
Paracetamol:	Isolated hepatocytes		+
	In vivo[1]		+
NAPQI[2]:	Isolated hepatocytes	+	

1. Data from ref 12
2. Reactive metabolite of paracetamol

systems tested and cause severe depletion of intracellular GSH. This depletion generally preceedes cytotoxic effects. Coincubation of smoke condensate with cultures of normal human bronchial fibroblasts and epithelial cells resulted in dose-dependent cytotoxicity as assessed by reductions in their respective colony forming efficiencies (CFE). When NAC was coincubated, this was shown to significantly protect both cell types from condensate induced reductions in CFE (10). In addition to this, the condensates also initiated terminal differentiation of the epithelial cells, as assessed both morphologically and by reductions in the dividing potential, or clonal growth rate of the cells. Once again, coincubation of NAC (10 mM) was shown to significantly protect cells from these effects.

The mechanism of the protective effect(s) of NAC in these cellular experiments was most probably due to direct interaction with acrolein and the reactive components of the cigarette smoke (Table I). The protective effects were de-

pendent on the NAC concentration and not dependent on a facilitated GSH biosynthesis as buthionine sulfoximine (BSO), a gluthathione synthesis inhibitor did not decrease the protective effect of NAC.

The situation is however different if a more physiological system, the isolated perfused ventilated rat lung is used. Cigarette smoke administered directly to the lungs endotracheally caused a rapid and dose-dependent depletion of total lung GSH (10). Also in this system, NAC was protective and if included in the perfusion medium, only limited GSH loss could be observed. This protective effect appears to be completely dependent upon stimulated GSH biosynthesis as the inclusion of BSO in the perfusion medium completely removed this protective effect (Table II). In addition, it seems unlikely that NAC in the perfusion medium could react directly with smoke constituents in the airways. Further more GSH present in the perfusion medium also had a protective effect. These results were expected since the rat lung has the ability to take up GSH (11).

Together these studies show that NAC protects against the adverse effects of cigarette smoke irrespective of the test system used. The mechanism of protective effect however appears to vary depending on the experimental system used. In extrapolating to the in vivo situation, a mechanism involving facilitated GSH biosynthesis seems most likely.

The protective effect of NAC against paracetamol toxicity is also mediated through a facilitated GSH biosynthesis as has been shown both in isolated hepatocytes (6) and in vivo (12) (Table I). However, if the reactive paracetamol product N-acetyl-p-benzoquinone imine (NAPQI) is added to isolated

TABLE II
PROTECTIVE EFFECT OF N-ACETYLCYSTEINE AGAINST CIGARETTE SMOKE INDUCED GSH LOSS - EFFECT OF BUTHIONINE SULFOXIMINE

Additions	nmol GSH/g tissue
None	917 ± 112
Cigarette smoke (4 cig.)	255 ± 81
Cigarette smoke + NAC (1 mM)	654 ± 73
Cigarette smoke + NAC + BSO (0.5 mM)	142 ± 55
Cigarette smoke + GSH (1 mM)	795 ± 68

Depletion of intracellular GSH was achieved by intracellular administration of the smoke from four cigarettes. Subsequent perfusion was performed as described in (11).

hepatocytes the mechanism is different. In this situation NAC is able to interact directly with NAPQI extracellularly and inactivate this reactive metabolite (13) (Table I).

NAC also interacts with free radicals and hydroperoxides. The interaction of NAC as well as GSH with free radical intermediates is not enzyme catalyzed. Free radicals can be either organic free radicals, as those found in cigarette smoke or formed as a consequence of metabolic activation of certain exogenous compounds, or oxygen free radicals i.e. superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$). The thiol dependent nonenzymatic detoxication of free radicals in vivo has not been well characterized but is probably of considerable physiological importance.

When studied in in vitro systems, the interaction of NAC and GSH with free radicals has been shown to result in the formation of the respective thiyl radical (14). The fate and the possible reactivity of the thiyl radicals is presently unclear but the major end product appears to be the disulphide. The formation of the disulphides does however not occur primarily by simple dimerization but is through a reaction dependent upon oxygen involving the intermediate formation of the sulphenyl hydroperoxide (15).

Active oxygen species include the free radical intermediates $O_2^{\cdot-}$ and $^{\cdot}OH$ as well as the nonradical hydrogen peroxide (H_2O_2). Whereas $O_2^{\cdot-}$ and H_2O_2 are relatively unreactive, $^{\cdot}OH$ is extremely reactive and has been shown to induce numerous adverse reactions in biological systems such as lipid peroxidation, DNA strand scission and enzyme inactivation.

Both NAC and GSH will react with all the reactive oxygen species. The interaction between $O_2^{\cdot-}$ formed by the xanthine/xanthine oxidase system and GSH has been shown to result in the formation of the glutathionyl radical (16). This was demonstrated by EPR spectroscopy using the spintrap DMPO. The physiological importance of the nonenzymatic interaction between $O_2^{\cdot-}$ and GSH or NAC is however doubtful due to the relatively low reactivity of $O_2^{\cdot-}$ (16). Concersely, these thiols interact much more readily with both $^{\cdot}OH$ and H_2O_2. Hydrogen peroxide is probably quantitatively the most important endogenous active oxygen species. It is a product of many endogenous enzymatic reactions, it is readily formed from $O_2^{\cdot-}$ by dismutation and may, in the presence of transition metals, form hydroxyl radicals (Fenton reaction).

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\cdot}OH + {}^{-}OH + Fe^{3+}$$

In the cell H_2O_2 is metabolized mainly by catalase and the selenium dependent enzyme glutathione peroxidase. Glutathione peroxidase has high affinity for H_2O_2 and efficiently reduces it to water with the concomitant formation of

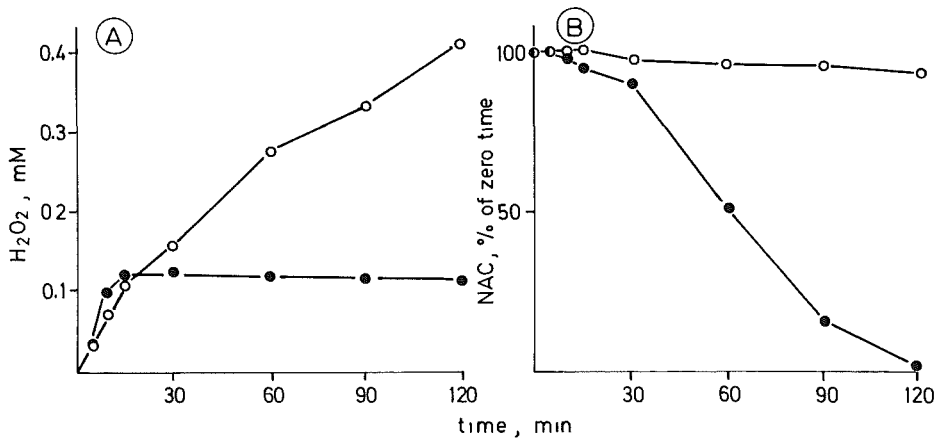

Fig. 1. Interaction of H_2O_2 derived from glucose-glucose oxidase with NAC (1 mM). Fig. 1a shows H_2O_2 concentration and Fig. 1b the effect on the concentration of NAC. o-o, H_2O_2 generating system; ●-● H_2O_2 generating system + NAC. Values are means from three experiments. For experimental details see ref. 19.

glutathione disulphide (GSSG). No glutathionyl radicals are formed in this reaction. The GSSG is rapidly reduced back to GSH at the expense of NADPH by glutathione reductase. Glutathione peroxidase is not specific for H_2O_2 but also metabolizes other hydroperoxide species, particularly lipid hydroperoxides, which may be formed as a consequence of lipid peroxidation or by lipoxygenase catalyzed reactions.

NAC is not a substrate for glutathione peroxidase and the interaction between H_2O_2 and NAC is nonenzymatic. NAC does indeed reduce H_2O_2 with the concomitant formation of the NAC disulphide. This interaction can be demonstrated using the H_2O_2 generating system glucose/glucose oxidase (Fig. 1).

As is evident from the results presented in this figure NAC interacts with the H_2O_2 formed but only after the H_2O_2 has reached a certain concentration. Thus the interaction between NAC and H_2O_2 appears to be of limited affinity. Naturally if NAC is present in high enough concentrations it protects cells against GSH depletion and toxicity induced by H_2O_2 (Fig. 2), presumably by a reaction with H_2O_2 extracellularly. Thus, in vivo, if sufficiently high concentration of NAC could be attained at, for example, an inflammatory site, NAC could scavenge H_2O_2 and/or other active oxygen species and act as a direct antioxidant.

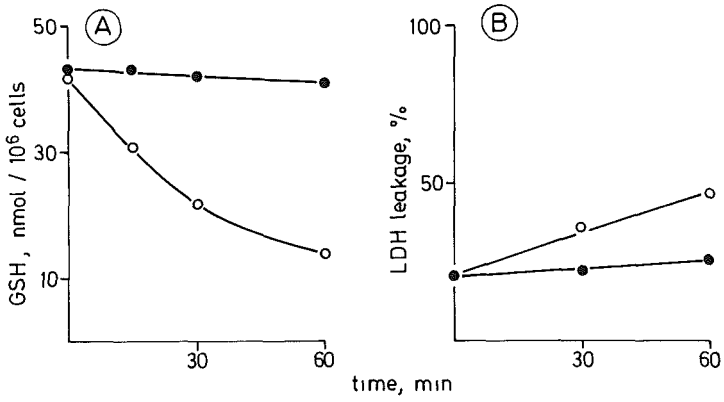

Fig. 2. Protective effect of NAC (1 mM) against H_2O_2 induced depletion of GSH (A) and toxicity (B) in isolated hepatocytes pretreated with BCNU. o-o H_2O_2 (2 mM); ●-● H_2O_2 + NAC.

If the generation of reactive oxygen species occur intracellularly NAC does however, only have a very limited protective effect. Formation of reactive oxygen species intracellularly can be achived utilizing a substrate which undergoes redox cycling. Such a compound in the bipyridylium compound diquat. In isolated hepatocytes diquat undergoes rapid redox cycling with concomitant formation of $O_2^{\cdot-}$ and concomitantly H_2O_2 and probably also $^{\cdot}OH$ and singlett oxygen. However, in normal hepatocytes diquat is not toxic (17). The reason for this lack of toxicity is related to the very effective H_2O_2 metabolizing system in hepatocytes. The glutathione peroxidase/glutathione reductase system appears to be the most important for the metabolism of H_2O_2 formed during diquat redox cycling. One may thus make the hepatocytes much more sensitive by inhibiting the glutathione reductase with pretreatment with 1,3-bis(2 chlorethyl(-1-nitrosourea (BCNU). In this compromized system diquat rapidly depletes cellular GSH levels with a concommitant GSSG formation and causes considerable cell death already after 90 min of incubation (17) (Table III). The mechanism of diquat induced toxicity is not resolved but these and more recent results using the iron chelator desferrioxamin indicate the importance of H_2O_2 and transition metals, presumably involved in the concommitant formation of $^{\cdot}OH$, in this type of toxicity.

TABLE III
DIQUAT (DQ) INDUCED GSH DEPLETION AND CYTOTOXICITY IN ISOLATED HEPATOCYTES

	GSH (nmoles/10^6 cells)		Toxicity (% trypan blue uptake)	
	t0'	t15'	t0'	t60'
Control	50	46	6	10
Control + DQ	55	50	6	20
+ BCNU	42	40	18	25
+ BCNU + DQ	40	6	18	80

Experiments were performed as described in ref 17.

The addition of NAC to incubations with diquat did not protect the compromised hepatocytes against the cytotoxicity. However, in the presence of the seleno organic compound, Ebselen (2-phenyl-1,2-benzoisoselenazyl-3(H)-one; PZ51), which possesses glutathione peroxidase activity (18) and also has been shown to be antiinflammatory, NAC protected against diquat induced GSH loss, lipid peroxidation and loss of cell viability (19) (Table IV). Ebselen alone did not have any significant effect on GSH levels or cell viability but inhibited diquat-associated lipid peroxidation (19).

TABLE IV
GSH AND GSSG LEVELS, LIPID PEROXIDATION AND VIABILITY OF BCNU TREATED HEPATOCYTES INCUBATED WITH DIQUAT IN THE PRESENCE AND ABSENCE OF NAC AND EBSELEN.

	GSH	GSSG	MDA	Cell death
	nmol after 15'		nmol after 90'	% after 90'
Control	38.5	-	3.5	29
DQ, 1 mM	4.2	19.5	10.1	82
+ NAC, 2x1 mM	7.0	14.3	14.5	69
+ NAC + Ebselen (50 uM)	21.5	6.0	3.5	31

Data from ref 19.

The mechanism of the protective action of Ebselen and NAC against GSH oxidation and toxicity induced by diquat redox cycling is not known and the reactions may occur either extra- och intracellularly. It is of course tempting to speculate about the possible therapeutic efficacy of Ebselen in combination with NAC in instances where rapid H_2O_2 production can be correlated with irreversible tissue damage, for instance during inflammatory processes or during redox cycling of environmental chemicals. We are awaiting further studies in this area.

CONCLUSION

NAC has the ability to scavenge a wide range of reactive and potentially toxic species. This interaction occurs nonenzymatically and is dependent on the concentration of NAC. NAC may also facilitate the biosynthesis of GSH efficiently in many organs.

The mechanism of NAC as a scavenger in vivo probably depends on its route of administration. Only when NAC is administred by inhalation or i.v. may a high enough concentration of NAC be reached in order to efficiently interact directly. An action through facilitated GSH biosynthesis seem more probable when NAC is administred orally.

Whether or not a scavenging effect of NAC is involved in the mechanism of action of NAC in lung related diseases is not known. Further more its possible use as a protective agent against the toxic effects of environmetntal chemicals or during inflammatory processes remains to be established.

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Discussion - Molecular scavenging: a new use for old drugs.

L.F. Prescott

Can you comment on the possible use of N-acetylcysteine in paraquat lung toxicity?

P. Moldeus

N-acetylcysteine alone does not protect against paraquat toxicity. Actually the effects of paraquat in the lung are due to a mechanism that seems different from that causing diquat hepatotoxicity. However, I believe that a study is under way to evaluate the efficacy of N-acetylcysteine + Ebselen.

J.V. Castell

Have you ever studied the effects of concentrations of diquat below 1 mmol? In our hands it is very difficult to see toxic effects on hepatocytes below this concentration.

P. Moldeus

Yes, and we got positive results. You should take into account that we use a system that incorporates inhibition of glutathione reductase, thus rendering the hepatocytes very sensitive to diquat toxicity.

J.V. Castell

Another question concerns alpha$_1$ antitrypsin activity. Have you studied if N-acetylcysteine exerts a protective effect on this protein in smokers?

P. Moldeus

Again, I cannot recall exact data, but I believe that some experiments have been done showing some protective effect of N-acetylcysteine on alpha$_1$ antitrypsin, in vitro.

P.G. Watanabe

Has Ebselen been studied in vivo?

P. Moldeus

Yes. This agent shows some antiinflammatory activity in

animals. The doses used are small and no toxicity is apparent. It does not release selenium. However, I do not know of any study in which it has been used together with N-acetylcysteine in vivo.

H. Vainio

Do you know of any other selenium compounds that share Ebselen effects?

P. Moldeus

No, I do not.