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THE METABOLISM OF *N*-ACETYLCYSTEINE BY HUMAN ENDOTHELIAL CELLS

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Abstract—When human umbilical endothelial cells were depleted of their glutathione by incubation in a sulfur amino acid-free medium, subsequent incubation of the cells with this deficient medium supplemented with *N*-acetylcysteine resulted in a dose-dependent stimulation of the synthesis of cellular glutathione. Similarly, the inclusion of *N*-acetylcysteine in the medium during the period of depletion of glutathione caused a dose-dependent retardation of the depletion kinetics. In contrast, the incubation of control cells in normal medium supplemented with *N*-acetylcysteine did not increase cellular glutathione levels above controls. These observations indicate the presence of an *N*-deacetylase in/on the cells with specificity for *N*-acetylcysteine. Due to the large surface area of the endothelium in the vasculature it seems likely that endothelial cell N-deactylation plays a role in the metabolic disposition of *N*-acetylcysteine, particularly when administered intravenously. *N*-Acetylcysteine is, however, a relatively poor precursor to glutathione biosynthesis in comparison to cystine. Thus, any cytoprotective, antioxidant effect exerted by *N*-acetylcysteine on the human endothelium is likely to be due to direct scavenging of reactive intermediates rather than by stimulated glutathione synthesis in the endothelial cells themselves.

The selective barrier function and metabolism of the endothelium of the blood vasculature plays a vital role in the control of tissue homeostasis. Destruction of the endothelium or impairment of endothelial function presents serious consequences to the organism. Such situations may occur in association with increased oxidative metabolism in and around the endothelium. Thus, endothelial cells, particularly those present in the major capillary network of the lung, have been shown to be sensitive to oxidative insult both *in vivo* and *in vitro*. The source of the oxidative insult may vary from exposure to oxidant gases such as NO_2 and O_3 [1, 2], to exposure to high partial pressures of oxygen [3] and to reactive metabolites (ROMs), such as $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$, generated during reperfusion of ischemic tissue [4] or by the oxidative burst of activated polymorphonuclear leukocytes (PMLs) [5].

It is now well established that both the intracellular and extracellular environments are well protected against the consequences of normal oxidative metabolism and temporary situations of increased oxidant burden [6]. The endothelial cell seems to be no exception to this, possessing an extensive network of both soluble and membrane-associated antioxidant principles [7]. One of the central components of the intracellular antioxidant network is the nucleophilic tripeptide glutathione (GSH) and the activity of related enzymes such as the glutathione peroxidases (GSHpxs) and glutathione reductase (GSHred) [8]. This system is responsible for the concerted detoxication of ROMs such as H_2O_2 and products of their interaction with cellular macromolecules, such as lipid hydroperoxides [9].

Glutathione-dependent antioxidant mechanisms may be of particular importance to the human endothelium as comparative studies with bovine cells have shown that human endothelial cells, such as those isolated from umbilical chords (HUVE cells) lack catalase activity [10, 11]. Indeed, experiments have shown that endothelial cells depleted of GSH are extremely sensitive to oxidative insult [12]. Conversely, attempts have been made to design chemical protective agents which augment the function of GSH. These agents may roughly be divided into two groups; agents which compete with GSH for chemical reaction with electrophiles, and agents which augment the activity of GSH by supporting its intracellular biosynthesis. An example of the latter case is the cyclic cysteine precursor oxathioxolidine (OTZ), which has been shown to elevate intracellular GSH levels in bovine pulmonary endothelial cells and concurrently increase their resistance to oxidative insult [12].

An example of an agent which may potentially fill both of the above protective roles is the thiol drug *N*-acetylcysteine (NAC). This molecule is used both orally and intravenously in a variety of clinical therapies, including in the treatment of chronic lung diseases such as bronchitis [13] and as an antidote to paracetamol poisoning [14]. This drug has also proved to be efficacious in relatively high intravenous doses in the treatment of a variety of experimental pathologies resembling human disease states which exhibit endothelial toxicity as a result of oxidative stress, including acute pulmonary oxygen toxicity [15] and septicemia and endotoxin shock [16]. Despite these observations *in vivo*, it is not certain how this thiol drug produces its protective effects, particularly at the endothelial level. As a typical thiol NAC is able to directly react with ROMs (i.e. is a "scavenger") [17] and may also act as a cysteine and GSH precursor following deactylation, which appears to be the major route of biotransformation

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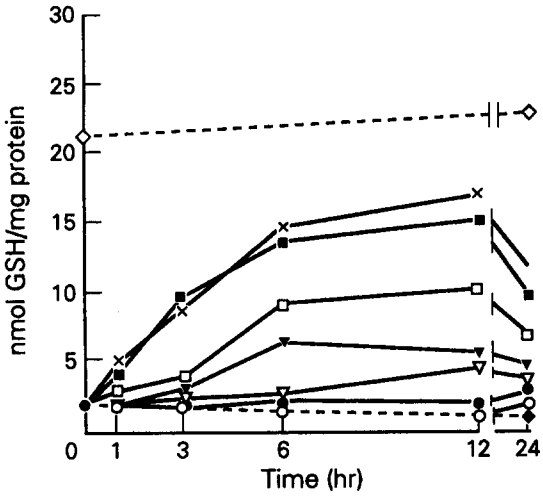

Fig. 1. The stimulatory effect of *N*-acetylcysteine on GSH synthesis in GSH-depleted HUVE cells. Cells were depleted of their GSH by an overnight incubation in sulfur-free medium M199 supplemented with 10% human serum. Cells were then supplied with fresh sulfur-free medium supplemented with NAC at various concentrations and cellular GSH determined as in the text. (◇---◇) Cells in complete medium; (◆---◆) Cells in M199(−) medium; (○——○) Cells in M199(−) medium; +5 μM NAC; (●——●) Cells in M199(−) medium; +10 μM NAC; (▽——▽) Cells in M199(−) medium; +25 μM NAC; (▼——▼) Cells in M199(−) medium; +50 μM NAC; (□——□) Cells in M199(−) medium; +100 μM NAC; (■——■) Cells in M199(−) medium; +500 μM NAC; (×——×) Cells in M199(−) medium; +1000 μM NAC.

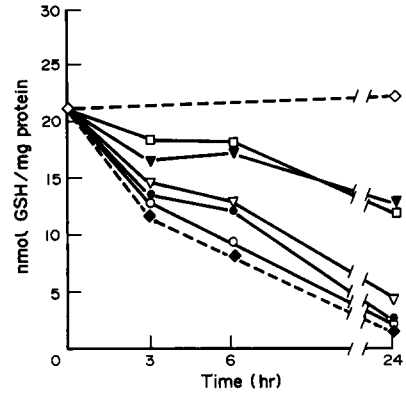

Fig. 2. The protective effect of *N*-acetylcysteine on the depletion of HUVE cell GSH by sulfur-free medium. Control cells were incubated in sulfur-free M199 medium supplemented with 10% human serum in the presence and absence of NAC. Samples were analysed from cellular GSH as in the text. (◇---◇) Cells in complete medium; (◆---◆) Cells in M199(−) medium; (○——○) Cells in M199(−) medium; +25 μM NAC; (●——●) Cells in M199(−) medium; +50 μM NAC; (▽——▽) Cells in M199(−) +100 μM NAC; (▼——▼) Cells in M199(−) medium; +500 μM NAC; (□——□) Cells in M199(−) medium; +1000 μM NAC.

[18]. Which of these components may contribute most to cytoprotection of the endothelium is unsure.

In this paper we report the ability of the human endothelium to metabolize NAC and utilize it as a metabolic precursor to GSH biosynthesis. The studies utilize human umbilical vein endothelial HUVE cells in culture [19] and employ GSH manipulation procedures recently described by us [20]. The data are discussed in terms of the human pharmacokinetics of NAC and the relative roles of stimulated GSH biosynthesis and direct scavenging in the protection of the endothelium during NAC administration *in vivo*.

MATERIALS AND METHODS

N-Acetylcysteine (>99%) was obtained from the Sigma Chemical Co., St Louis, MO, U.S.A.). *N*-Acetylcysteine disulfide was obtained by the oxidation of NAC as described previously [21]. All materials for the isolation and culture of HUVE cells were as referenced. Umbilical chords were obtained less than 6 hr post-parturitum from the Delivery center of Danderyds Hospital and the cells isolated by collagenase perfusion and placed in primary culture as described by Jaffe *et al.* [19], with slight modifications [20]. Cells were seeded onto

Table 1. The decline of *N*-acetylcysteine free thiol levels of spiked medium

Time (hr)	Medium NAC concentration (μM) (% control/hr)*						
0	11	23	47	99	256	479	1090
1	7 (36)	20 (13)	39 (17)	82 (12)	226 (12)	431 (10)	1085 (2)
3	6 (15)	16 (10)	31 (11)	80 (6)	200 (7)	402 (5)	864 (7)
6	4 (11)	13 (7)	26 (8)	61 (6)	171 (6)	328 (5)	731 (5)
24	1 (4)	5 (3)	15 (3)	30 (3)	62 (3)	125 (3)	359 (3)

N-Acetylcysteine was incubated in sulfur-free M199 medium supplemented with 10% human serum under the same conditions as for cell experiments. Samples were removed and analysed for free NAC by HPLC as described in Materials and Methods.

* Calculated as:

$$\frac{\text{Start concentration} - \text{test concentration at time } t}{\text{Start concentration}} \times 100 \times \frac{1}{t}(\%/\text{hr}).$$

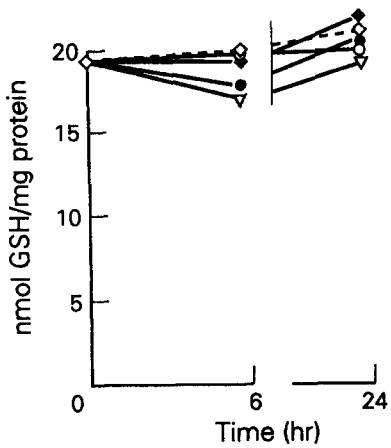

Fig. 3. Nil effect of *N*-acetylcysteine on control HUVE cell GSH levels. Control cells were incubated in complete medium with NAC and the effect on cellular GSH levels determined. (◇---◇) Cells in complete medium; (◆---◆) Cells in complete medium; + 50 μM NAC; (○——○) Cells in complete medium; + 100 μM NAC; (●——●) Cells in complete medium; + 250 μM NAC; (▽---▽) Cells in complete medium; + 500 μM NAC.

35 mm dishes and used at the confluency of the third passage throughout. The endothelial nature of the cells was confirmed by their cobblestone morphology and the expression of factor viii antigen by immunofluorescence (data not shown).

For some experiments cells were depleted of their GSH by an overnight (18 hr) incubation in a medium free of any low molecular weight sulfur-containing components, e.g. cystine, cysteine, GSH and methionine. Although the medium was supplemented with 10% human bank serum, the serum does not provide any low molecular thiols due to their oxidation and mixed disulfide formation with serum proteins during storage [20]. Similarly, any serum methionine will not influence the depletion as HUVE cells lack the activity of the cystathionase pathway [20]. Depleted and control cells were incubated with NAC in various media and at various concentrations of the drug and the cellular GSH analysed at various times.

For the analysis of cellular thiols the cells were washed with PBS supplemented with 1% BSA (4 × 1 mL) and thiols derivatized *in situ* with the membrane-permeable, thiol-reactive agent monobromobimane (mBBr), separated by reversed-phase liquid chromatography and quantitated by fluorescence detection [22]. Similar determinations were made on media samples for determination of the control oxidation of NAC [21]. Cellular protein was determined by the method of Peterson [23].

RESULTS AND DISCUSSION

When HUVE cell GSH was depleted by incubation of the cells in a sulfur amino acid-free medium overnight, a manipulation previously shown to allow rapid resynthesis of the tripeptide with the prerequisite precursors [20], resupply of NAC as the only sulfur source in the medium facilitated resynthesis of GSH at levels above 25 μM in the medium (Fig. 1). At no time were free NAC or cysteine detected in the cells treated with NAC under these conditions. This threshold concentration is well above the lowest concentration of cystine and cysteine (3–5 μM each) which will stimulate GSH biosynthesis in these cells under similar circumstances [20]. This discrepancy may be dependent on two interacting parameters. Firstly, it may be that these concentrations of NAC are well below the K_m of the deactylation step which is obligatory in the liberation of cysteine for support of GSH synthesis. Secondly, like all thiols NAC is susceptible to oxidation and control experiments showed that the free thiol disappeared from the medium by sigmoidal kinetics. It can be seen from Table 1 that the initial decline in free NAC (0–3 hr) was rapid and dose-dependent, being most pronounced below 25 μM. Thus, at the lower concentrations of the drug (i.e. under 25 μM) it may be that this oxidation is too rapid and extensive for sufficient deactylation to occur. It will be noted that an authentic sample of NAC disulfide failed to support any GSH biosynthesis under these conditions, perhaps indicating a lack of affinity of this species for the deactylase enzyme.

A combination of these two factors may also be responsible for the time-lag of onset of synthesis of GSH stimulated by NAC and the apparent inefficiency of NAC as a precursor in comparison to cystine or cysteine. Even with the highest concentration of NAC tested (1 mM) the accumulation of cellular GSH was far inferior to that achieved with optimal concentrations of these endogenous sulfur-containing amino acids (25–50 μM) [20]. Furthermore, all concentrations of the drug which were stimulatory to GSH synthesis resulted in a peak accumulation between 6–12 hr and a gradual decline up to 24 hr. This is diagnostic of lack of precursor supply and may be explained by the oxidation kinetics of NAC in the medium, especially at the lower concentrations of the drug (Table 1).

In order to test the efficiency of NAC as a precursor to HUVE cell GSH synthesis under conditions of "pressure" on cellular levels of the tripeptide, we coincubated cells with sulfur-free M199 medium with and without the supplementation of NAC as sole sulfur source. It can be seen in Fig. 2 that NAC protected cells from the depletory effect of the medium in a dose-dependent manner. However, it is again evident that NAC is a relatively poor precursor to GSH biosynthesis, even at concentrations above 500 μM, where there was a failure to maintain levels at >60% of controls by 24 hr of incubation. Additionally, Fig. 3 shows that when control cells were incubated in complete medium supplemented with NAC up to 1 mM no elevation of cellular GSH was noted. Again, during both of these series of experiments, neither cysteine nor free NAC were detected in the cells.

Taken in combination with the established pharmacokinetic data for the drug, these metabolic observations have important ramifications for the interpretation of the mechanism of NAC's protective

effects on the endothelium in a number of oxidative stress-associated pathologies. It is unlikely that either single or repeated oral doses of the drug, according to the clinically-accepted dosing regimes, i.e. up to 4 × 400 mg daily, will support novel synthesis of GSH in the resting or "stressed" human endothelium as the peak plasma level of the free drug does not exceed 10 μM [21, 24–26]. This seems to be due to the extensive first pass metabolism of the compound [18] and rapid oxidation to disulfide species in the plasma [21, 24, 26]. Thus, it is unlikely that oral dosing of NAC will have any protective potential in the human endothelium either by supporting intracellular GSH levels or by acting as a direct scavenger for ROMs. When the drug is administered by intravenous infusion or injection, where the plasma levels of the free thiol exceed 200 μM at peak, it is likely that the relatively poor efficacy of the drug as a precursor to endothelial cell GSH synthesis ensures that any beneficial effects on oxidant-mediated endothelial toxicity occur as a result of direct scavenging of ROMs by the thiol.

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