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Original Contribution

N-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress

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Abstract

Oxidative stress plays an important role in the progression of neurodegenerative and age-related diseases, causing damage to proteins, DNA, and lipids. A novel thiol *N*-acetylcysteine amide (AD4), the amide form of *N*-acetylcysteine (NAC) and a Cu^{2+} chelator, was assessed for its antioxidant and protective effects using human red blood cells (RBCs) as a model. AD4 was shown by flow cytometry to inhibit *tert*.-butylhydroxyperoxide (BuOOH)-induced intracellular oxidation in RBCs stained with the oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate. In addition, AD4 retarded BuOOH-induced thiol depletion and hemoglobin oxidation. Restoration of the thiol-depleted RBCs by externally applied AD4 was significantly greater compared with NAC and, unlike NAC, was accompanied by hemoglobin protection from oxidation. In a cell-free system we have demonstrated that AD4 reacted with oxidized glutathione (GSSG) to generate reduced glutathione (GSH). The formation of GSH was determined enzymatically using GSH peroxidase and by HPLC. Based on these results a thiol–disulfide exchange between AD4 and GSSG is proposed as the mechanism underlying the antioxidant effects of AD4 on BuOOH-treated RBCs. Together, these studies demonstrate that AD4 readily crosses cell membranes, replenishes intracellular GSH, and, by incorporating into the redox machinery, defends the cell from oxidation. These results provide further evidence for the efficient membrane permeation of AD4 over NAC, and support the possibility that it could be explored for treatment of neurodegeneration and other oxidation-mediated disorders.

Keywords: Oxidative stress; AD4; Free radicals; Neurodegeneration; Glutathione; Antioxidant *N*-acetylcysteine amide; Human red blood cells

Introduction

In view of the redox regulation of major cellular events (e.g., metabolism, gene expression, signal transduction), attempts to protect cells by exogenous redox active agents have intensified [1,2]. Thiol-containing compounds have earned special attention due to the profound role played by intracellular glutathione in antioxidant cell defense and redox regulation. Depletion of reduced glutathione (GSH), indicating a decrease in the antioxidant capacity of the cell, has been associated with aging, diabetes mellitus, inflammation, and neurodegenerative diseases [3–5].

Supplementing cells with exogenous thiol agents has been used to replenish GSH. In vitro, both dithiothreitol (DTT) and *N*-acetylcysteine (NAC) augmented intracellular thiols in endothelial cells and prevented apoptosis induced by lipopolysaccharides or arsenate [6]. In human red blood cells (RBCs), exogenous NAC, but not GSH, partially restored intracellular thiols depleted by various oxidants [7]. In sheep RBCs, exogenous DTT, but not GSH, protected

Abbreviations: AD4, *N*-acetylcysteineamide; BuOOH, *tert.*-butylhydroperoxide; BuOH, *tert.*-butanol; $BuOO^{\bullet}$, *tert.*-butylperoxyl; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DTNB-5,5, dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; G-6-P, glucose 6-phosphate; G-6-PD, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GPx, glutathione peroxidase; GSH, glutathione (reduced); GSSG, glutathione disulfide; Hb, hemoglobin; NAC, *N*-acetylcysteine; RBC, red blood cells.

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against cell oxidation induced by *tert*.-butylhydroperoxide (BuOOH); the protection required functional glutathione peroxidase (GPx) [8].

In vivo, however, the clinically available agent NAC failed to provide significant antioxidant effects, presumably due to its low lipid solubility and tissue distribution [9]. Among several newly developed agents with improved pharmacokinetic properties, γ-glutamyl-cysteinyl-ethyl ester was shown to cross the blood–brain barrier and increase the total GSH pool in rat brain [5]. The carboxyl esterification made this dipeptide more lipophilic which greatly increased its tissue distribution and biological activity. Likewise, diethyl esterification of glutathione facilitated its transport into human cells including RBCs [10].

This strategy of drug design and the potential benefits of replenishing intracellular thiols led one of us to propose a new series of lipophilic thiol compounds able to cross the cell membrane [11]. In NAC, the carboxyl group is negatively charged at physiological pH, limiting the drug's ability to cross cell membranes. A newly designed amide form of NAC, *N*-acetylcysteineamide (AD4), in which the carboxylic group is neutralized, is expected to be more lipophilic and cell-permeating [11]. This compound was recently shown to cross the blood–brain barrier, scavenge free radicals, chelate copper ions (Cu^{2+}), and protect mice from MOG-induced clinical symptoms known as experimental autoimmune encephalomyelitis, used as an animal model of multiple sclerosis [12].

For the present report, we studied the reducing properties, cell permeation, cytoprotective effects, and mechanism of action of AD4. We chose human RBCs as a model because these readily available cells contain the complete enzymatic machinery of GSH oxidation, reduction, and synthesis. This allowed us to study AD4 effects on intracellular thiol balance under various oxidation conditions. Below, we demonstrate strong antioxidant and protective effects of AD4 on RBCs most likely due to its capability to cross the cell membrane and replenish intracellular GSH. We demonstrate that the mechanism underlying AD4 activity could largely be attributed to a direct reduction of GSSG to GSH.

Materials and methods

BuOOH, H_2O_2

DTNB (5, 5–dithiobis-2-nitrobenzoic acid), NAC, glutathione (both oxidized and reduced), glutathione peroxidase (EC 1.11.1.9), glutathione reductase (EC 1.6.4.2), and other essential chemicals were purchased from Sigma, Israel. All stock solutions were treated with iron-binding beads (Chelex, Sigma) to diminish adventitious iron-catalyzed reactions.

Thiol reactivity toward H_2O_2 and BuOOH was determined by incubating the tested thiol (AD4, NAC, or GSH) at 1 mM with either oxidant at 0.5 mM in PBS at pH 7.4 and 37°C for 10 min. This ratio of 2:1 (thiol:peroxide) is stoichiometric for monothiols reacting with H_2O_2 or BuOOH (see reaction 3 and [13,14]). The thiol concentration at a given time point of the reaction was measured as specified below and the results were fitted by the second-order kinetics according to the equation

$$1/C_t - 1/C_0 = Kt, \tag{1}$$

where C_0 and C_t are the concentrations at 0 and t time points, and K is the second-order rate constant.

Enzyme catalyzed oxidation of the tested thiols with BuOOH was carried out in the presence of either commercial glutathione peroxidase (GPx) from bovine RBCs or lysates of human RBCs as indicated in the legend to Fig. 6. The reaction was initiated by the addition of BuOOH (0.5 mM) and stopped at various time points by acidifying the reaction mixture with 1% (final) metaphosphoric acid. The enzyme-catalyzed thiol oxidation was analyzed using the parameters of first order kinetics [15],

$$-\ln(C_0 - C_t) = kt, \tag{2}$$

where k is the first-order rate constant. Linear regression analysis of experimental points was then applied to obtain the numerical values of the rate constants.

RBCs were prepared from hematologically normal blood samples counted on the same day using a Coulter cell counter. Plasma and buffy coat were removed, and RBCs were washed three times in PBS-1(136 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4). Final cell concentrations were calculated using microscopic cell count or hematocrit determination.

The RBC suspension (10%) was incubated with various additions in PBS-2: 100 mM NaCl, 0.5 mM EDTA, 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.4 at 37°C. The higher buffer capacity of PBS-2 provided pH stability in the presence of millimolar concentrations of exogenous thiols. EDTA prevented free iron from entering the cells.

Nonprotein thiols were measured using a procedure adapted and modified from Beutler [16]. RBC samples were washed in PBS-1 and lysed by adding 10 vol of ice-cold 5 mM Na_2HPO_4/NaH_2PO_4 pH 7.1, containing 1 mM EDTA. An aliquot of lysate (150 μl) was mixed with 225 μl of ice-cold precipitating solution (1.67% *meta*-phosphoric acid, 30% NaCl, and 1 mM EDTA). After 10 min on ice, the precipitated material was separated by centrifugation. For thiol determination, 200 μl of the acidic extract was neutralized with 800 μl of 0.3 M Na_2HPO_4, and then 100 μl of 0.5 mM DTNB was added. Absorbance of the resulting monothiol–TNB was determined at 412 nm, and its concentration was calculated using the extinction coefficient $\varepsilon = 13.7\ mM^{-1}\ cm^{-1}$. This concentration represented the total amount of reactive nonprotein thiols in a given RBC sample.