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Research paper

A thioredoxin reductase and/or thioredoxin system-based mechanism for antioxidant effects of ambroxol

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ABSTRACT

Long-term treatment with ambroxol (ABX), a bronchial expectorant, was found to prevent acute exacerbation of chronic obstructive pulmonary disease (AECOPD). The underlying mechanism remains unclear. To address this, we have investigated the effect of ABX on critical antioxidant proteins thioredoxin (Trx) and thioredoxin reductase (TrxR) that are decreased in patients with AECOPD. Trx, TrxR and NADP(H) form Trx system, which is involved in regulating numerous oxidative stress-related events. In human bronchial epithelial cells, treatment with ABX from 0 to 200 μM gradually increased mRNA and protein levels of TrxR/Trx. At these ABX concentrations, TrxR activity was elevated progressively, whereas Trx activity exhibited a dose-dependent biphasic response, increasing at 50 and 75 μM, but decreasing at ABX over 150 μM. Pre-treatment with 75 μM ABX enhanced the capacity of the cells to eliminate reactive oxygen species, which was largely prevented by knockdown of cytosolic Trx (hTrx1). In a purified system, ABX shortened the initial lag phase during the reduction of insulin disulfide by Trx system. Pre-treatment of NADPH-reduced TrxR with ABX caused a dose- and time-dependent increase in thiolate/selenolate species, i.e. the catalytically active form of TrxR. Kinetic analysis demonstrated that the reduction of H_2O_2 by TrxR or Trx system were enhanced by 100 or 200 μM ABX. When hTrx1 was mixed with ABX in a molar ratio of 1:1 to 1:100 (which could occur in human plasma), changes in intrinsic Trp fluorescence occurred, and the response of reduced hTrx1 was especially remarkable. These data reveal an ABX-sensing mechanism of TrxR/Trx. We therefore conclude that the antioxidant actions of ABX at physiological concentrations are, at least partially, mediated by TrxR and/or Trx system.

1. Introduction

Ambroxol [trans-4-(2-amino-3,5-dibromobenzylamino)-cyclohexanol, ABX (Fig. 1)] is a bronchial expectorant, mainly used in the treatment of respiratory diseases [1]. ABX may cross cell membrane [2], has a high affinity for lung tissue after oral or *i.v.* administration, and can achieve micromolar concentrations *in vivo* [3] with little toxicity [4–6]. Its ability to scavenge oxidants, like hydroxyl radical (˙OH), hypochlorite (HOCl), superoxide anion (O_2^-), and peroxidative metabolites reflected its importance in antioxidant defense [5,7–9]. Animal studies further tightened the association between ABX intake and antioxidant augmentation [8–10]. ABX is often used as an adjunct to conventional chronic obstructive pulmonary disease (COPD) treatments, and found to be useful in improving airways patency and preventing acute exacerbation of COPD (AECOPD) [11,12]. However, the underlying molecular mechanism is less understood.

Oxidative stress is an important contributor to the development of COPD [13,14]. It is quite interesting to know whether ABX could communicate with the antioxidant proteins that are involved in AECOPD. Recently, we have found that the patients with AECOPD have significantly low levels of serum thioredoxin (Trx) and thioredoxin reductase (TrxR), both of them are well-known antioxidant proteins [15]. In this context, we chose Trx/TrxR as model proteins to test the effect of ABX on their antioxidant capacity.

TrxR, Trx and NADPH compose Trx system. It is present in all living cells and critical to keep cellular redox balance [16,17]. Mammalian TrxR is a homo-dimeric FAD-containing selenoprotein. Compared to *Escherichia coli* TrxR, mammalian TrxR has a broad

Abbreviations: 5-IAF, 5-(iodoacetamido)fluorescein; ABX, trans-4-(2-amino-3,5-dibromobenzylamino)-cyclohexanol, ambroxol; DCFH-DA, 2′-7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorofluorescin; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; Gua HCl, guanidine hydrochloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMSF, phenylmethanesulfonyl fluoride; ROS, radical oxidative species; Trx, thioredoxin; TrxR, thioredoxin reductase.

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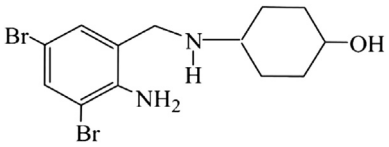

Fig. 1. Structure of ABX.

range substrate specificity. A unique C-terminal "–Gly-Cys-Sec-Gly" motif is essential for the catalytic activity of mammalian TrxR [18]. With a low pK_a and strong nucleophilicity, the Sec residue serves as a catalyst for electrons transfer to substrates *via* forming a reversible selenenylsulfide with the neighboring Cys residue [18,19]. Generally, substrate, such as oxidized Trx (Trx–S_2) or H_2O_2, is reduced into the reduced form [Trx–$(SH)_2$ or H_2O] by the selenothiol group in the reduced TrxR. Then, the oxidized TrxR with selenenylsulfide is re-reduced in the reaction with NADPH [18,20]. The reduced form of Trx can reduce a number of disulfide containing molecules [17], including ribonucleotide reductase, an essential enzyme for DNA synthesis and cell growth [21]. Besides Trx and H_2O_2, some other non-disulfide-containing molecules, such as selenite [22], lipid hydroperoxide [23] and dehydroascorbic acid [24], are also substrates for mammalian TrxR.

There are different isoforms of human Trx. The most well-studied one is cytosolic Trx (hTrx1) that contains five Cys residues. Of them, Cys32 and Cys35 participate in electrons transfer from TrxR to substrate *via* thiol–disulfide exchange reaction; Cys62 and Cys69 may form a second disulfide under oxidative stress [25]; Cys62 and/or Cys73 can be nitrosylated [26], and Cys73 located on the surface is the target for glutathionylation [27] or the site to form intermolecular disulfide [28]. hTrx1 homodimer is inactive [29]. Through transcriptional and post-translational modifications, hTrx1 has been found to be implicated in some signal transduction pathways and certain diseases [27,30]. For instance, changes of Trx expression were observed in lung at the early stage of COPD [31].

Here we evaluate the effect of ABX on cellular TrxR1/Trx1 and purified TrxR/Trx1. Our findings describe a TrxR/Trx-based mechanism involving in the ABX-initiated anti-oxidation effects.

2. Materials and methods

2.1. Proteins and chemicals

Mammalian TrxR was prepared from calf liver (referred as CL-TrxR) as described previously [32]. Recombinant hTrx1 was prepared according to the method described previously [33]. Monoclonal antibodies against hTrx1 and TrxR were purchased from Santa Cruz Biotechnology (Germany). β-Actin and GAPDH monoclonal antibodies were from Beyotime (China). Biotinylated secondary antibody and Streptavidin-ALP anti-biotin tertiary antibody were purchased from ZSGB-BIO (China). 4-Hydroxy-2-nonenal (4-HNE) was obtained from Calbiochem (USA) as a stock solution in ethanol. ABX, insulin, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dithiothreitol (DTT), guanidine hydrochloride (Gua HCl), phenylmethanesulfonyl fluoride (PMSF), NADPH and 5-(iodoacetamido) fluorescein (5-IAF) were purchased from Sigma (USA). Urea was from Amresco (USA). An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit and a Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit were from Invitrogen (USA).

2.2. Cell culture

Human bronchial epithelial cells (16HBE) were cultured in minimum essential medium (MEM, Invitrogen, USA) supplemented with 15% FBS (Xuri, China) and 100 μg/mL gentamycin sulfate (Amresco, USA) at 37 °C in an incubator containing 5% CO_2. To examine the effect of ABX, the cells were cultured until ~70% confluence, then incubated in the above-mentioned medium containing different concentrations of ABX for 24 h. After washes in PBS, the cells were harvested, and lysed with ultrasonication in the presence of 1 mM PMSF. Protein concentration was determined by BCA procedure using BSA as a standard. The cell protein extraction was frozen at −80 °C until used.

2.3. MTT cell viability assay

The cells were seeded into 96-well plates at a density of 5×10^4 cells per well. One–two days after seeding, the cells were exposed to different concentrations of ABX. After 24 h, MTT was added to each well at final concentration of 500 μg/mL, and plates were incubated at 37 °C for another 4 h. The culture medium in each well was then aspirated, and the resulting MTT–formazan product was dissolved by the addition of DMSO. Cell viability, which was proportional to the amount of formazan product, was determined by measuring the absorbance at 540 nm using the enzyme mark instrument (Multiskan MK3, Thermo, USA).

2.4. Measurement of cell proliferation

16HBE cells were seeded into 6-well plate and treated with indicated concentrations of ABX. A Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit was used to detect the newly synthesized DNA in cells, according to the manufacturer's protocol. The fluorescence was detected by flow cytometry (BD FACSCalibur, USA) and the data from 20,000 cells were collected and analyzed using CellQuest Pro Software.

2.5. Detection of hTrx1 redox state in cells

Thiol redox state of hTrx1 was detected using modified redox western blot developed from Refs. [34,35]. To prepare standards, cell lysates were denatured with 8 M urea and fully reduced with 3.5 mM DTT. Then varying molar ratios of iodoacetic acid (IAA) to iodoacetamide (IAM) were incubated with the reduced hTrx1 containing five cysteine residues, producing six hTrx1 isoforms with introduced number of acidic carboxymethyl thiol adducts (–SA^-) and neutral amidomethyl thiol adducts (–SM). During urea-PAGE, the ionized –SA^- adducts resulted in faster protein migration toward the anode. Therefore, the six isoforms were separated and used as a mobility standard for representing the number of –SA^-, indicating the different redox states of hTrx1. To prepare samples, 16HBE cells were seeded into 10-cm plates, treated with 75 or 200 μM ABX for 24 h and/or 100 μM H_2O_2 for 1 h, then washed twice with PBS and lysed in 300 μL of urea lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 8 M urea) containing 30 mM IAA. Free thiols were alkylated by IAA at 37 °C for 1 h. After removing the cell debris by centrifugation, the proteins from cell lysates were precipitated and washed by ice-cold acetone-HCl, then resuspended in 100 μL of urea lysis buffer containing 3.5 mM DTT. After incubation at 37 °C for 1 h, 10 mM IAM was added to each sample, and incubated for another 1 h at 37 °C. Protein concentration was determined by BCA assay. Equal amounts of protein were separated by urea-PAGE (12% gel) and electroblotted to a PVDF membrane (PALL, MI, USA). The membrane was probed with anti-hTrx1 primary antibody (diluted at 1:1000), biotinylated secondary antibody (diluted at 1:500) and Streptavidin-ALP anti-biotin tertiary antibody (diluted at 1:500), and then visualized by using a 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium substrate (Amresco, USA).