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Hypothiocyanous acid oxidation of tubulin cysteines inhibits microtubule polymerization

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ABSTRACT

Thiol oxidation is a probable outcome of cellular oxidative stress and is linked to degenerative disease progression. In addition, protein thiol redox reactions are increasingly identified as a mechanism to regulate protein structure and function. We assessed the effect of hypothiocyanous acid on the cytoskeletal protein tubulin. Total cysteine oxidation by hypothiocyanous and hypochlorous acids was monitored by labeling tubulin with 5-iodoacetamidofluorescein and by detecting higher molecular weight inter-chain tubulin disulfides by Western blot under nonreducing conditions. Hypothiocyanous acid induced nearly stoichiometric oxidation of tubulin cysteines (1.9 mol cysteine/mol oxidant) and no methionine oxidation was observed. Because disulfide reducing agents restored all the polymerization activity that was lost due to oxidant treatment, we conclude that cysteine oxidation of tubulin inhibits microtubule polymerization. Hypothiocyanous acid oxidation of tubulin cysteines was markedly decreased in the presence of 4% glycerol, a component of the tubulin purification buffer. Due to its instability and buffer- and pH-dependent reactivity, hypothiocyanous acid studies require careful consideration of reaction conditions.

Introduction

Our *in vitro* work with microtubule proteins including tubulin, tau and microtubule-associated protein-2 (MAP-2) shows that cysteine oxidation to disulfides by peroxynitrite anion, hypochlorous acid (HOCl)[1] and other oxidants is associated with decreased microtubule polymerization [1–3]. Tubulin, a heterodimer composed of similar 50 kDa α- and β-subunits, contains 20 reduced cysteines (12 in α-tubulin and 8 in β-tubulin) [4,5]. Because some tubulin cysteine oxidation (~1–2 mol cys) by oxidants is tolerated before microtubule polymerization is compromised, microtubule protein thiols may protect other cellular targets from oxidation [1,6]. This hypothesis is reinforced by our studies showing that the disulfides in tubulin and microtubule-associated proteins are repaired by the thioredoxin and glutaredoxin reductase systems thereby restoring polymerization activity [1,7].

Though present in all cells, tubulin constitutes 10–15% of total cellular protein in neurons [8,9]. Tubulin cysteine modifications including oxidation to disulfides, S-glutathionylation and S-nitrosation, have been identified in several proteomics studies using cell lines and tissue samples [10–13]. Recently, tubulin was identified as a target for thiol oxidation by HOCl and chloramines in endothelial cells [14].

In this study, we examine the effects of hypothiocyanous acid (HOSCN) on purified porcine tubulin. HOSCN is a cellular oxidant formed from thiocyanate ion (SCN^-) and H_2O_2 by peroxidases including myeloperoxidase (MPO) and eosinophil peroxidase [15,16]. HOSCN, like HOCl, oxidizes protein thiols and, if produced in cells, will likely affect tubulin cysteines [17,18]. Our current interest in HOSCN is twofold: (1) HOSCN is more selective for cysteines than other oxidants tested. Our published work over the past decade has included oxidants that cause additional types of damage–for example, methionine oxidation, S-nitrosation and tyrosine nitration [3,6,19]. While Angeli's salt is largely a cysteine oxidant, presumably via release of HNO, it produces nitrite as a byproduct which could yield nitrosation of cysteines. (2) MPO is aberrantly expressed in Alzheimer's disease brain [20,21]. Moreover, MPO immunoreactivity co-localized with neurofibrillary tangles in neurons of Alzheimer's disease brain and, 3-chlorotyrosine, a marker of HOCl oxidation was detected. Based on these findings, it is reasonable to hypothesize that HOSCN and HOCl could be formed by MPO in AD neurons.

Our focus on tubulin allows us to categorize oxidants with respect to their specificity for cysteine rather than other amino acids. Total cysteine oxidation and effects on microtubule polymerization by HOSCN and previously characterized oxidants, HOCl, chloramines and Angeli's salt, an HNO donor, are presented [3].

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[1] *Abbreviations used:* AS, Angeli's salt; BCA, bicinchoninic acid; DTT, dithiothreitol; GC, glycine chloramine; HNO, nitroxyl; HOCl, hypochlorous acid; IAF, 5-iodoacetomido-fluorescein; PB, phosphate buffer; PME, 0.1 M PIPES pH 6.9, 1 mM $MgSO_4$, 2 mM EGTA; TCEP, Tris(2-carboxyethyl)phosphine; TNB, thionitrobenzoic acid

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Materials and methods

Materials

Porcine brains were obtained from Smithfield Packing Company in Smithfield, Virginia. Angeli's salt was from Cayman Chemicals (Ann Arbor, MI). Bicinchoninic acid (BCA) protein assay reagent, West Pico chemiluminescence detection system, Tris(2-carboxyethyl)phosphine (TCEP) and 5-iodoacetomido-fluorescein (IAF) were from Thermo Pierce. The mouse anti-β-tubulin antibody (clone TUB 2.1) and the goat anti-mouse secondary antibody HRP conjugate were from Sigma. All other chemicals were from Fisher or Sigma. The concentration of HOCl was determined by measuring the absorbance at 292 nm ($\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$) in 0.1 M NaOH [22]. A solution of Angeli's salt was prepared immediately prior to use in 0.01 M NaOH and stored on ice. Glycine chloramine was prepared as described [3].

Preparation of hypothiocyanous acid

HOSCN was synthesized enzymatically in 0.1 M phosphate buffer pH 6.4. A typical reaction (250 μl) contained 12.5 μg LPO, 1.2 mM KSCN and H_2O_2. After 15 min at 22 °C, the reaction was quenched with 500 U catalase. HOSCN was separated from LPO and catalase using an Amicon Ultra centrifuge filter with a 10 K cutoff. HOSCN was stored on ice and its concentration was determined using thionitrobenzoic acid (TNB). Under these conditions, the concentration of HOSCN was 225–240 μM.

Purification of porcine brain tubulin

Tubulin was purified from porcine brain by two cycles of temperature-dependent polymerization and depolymerization and subsequent phosphocellulose chromatography as described [3].

Labeling of tubulin cysteines with IAF

Tubulin (6 μM, 120 μM cys) was diluted in either 0.1 M PB pH 7.4 or PME buffer pH 6.9 and then treated with each oxidant for 10 min at 22 °C in a total reaction volume of 10–20 μl. Either methionine or dichlorodimedone (200 μM) was added to quench high HOCl concentrations. IAF (1.2 mM) in DMF was added to achieve a 10-fold molar excess relative to protein cys and samples were incubated at 37 °C for an additional 30 min. Proteins were resolved by SDS–PAGE on 7.5% gels under reducing conditions and gel images were captured using a Bio-rad Chemi-doc XRS imaging system. The intensity of the fluorescein-labeled protein bands was measured using Bio-rad Image Lab software. Alternatively, IAF-labeled tubulin was precipitated with 80% ethanol, incubated on ice for 20 min and the protein pellet was collected at 16,000*g* for 20 min. Pellets were washed twice with 80% ethanol and then resuspended in 3 M guanidine HCl in 0.1 M Tris pH 8.8. Fluorescein in each protein sample was quantitated at 490 nm relative to a fluorescein standard curve prepared in 3 M guanidine HCl in 0.1 M Tris pH 8.8.

Detection of interchain disulfides by Western blot

Following treatment with oxidants as described above, tubulin species (10–20 μg protein per lane) were separated by SDS–PAGE on 7.5% polyacrylamide gels under nonreducing conditions. Proteins were transferred to PVDF membranes, blocked with 3% milk for 30 min and probed with a mouse monoclonal anti-β-tubulin antibody (1:2000) for two hours. The β-tubulin/antibody complex was detected using a goat anti-mouse HRP conjugate (1 h, 1:10,000) and Pierce West Pico chemiluminescent substrate. Chemiluminescence was captured using the Bio-rad Chemi-doc XRS imaging system.

Microtubule polymerization assays

Purified tubulin, diluted with PME or PB, was treated with up to 75 μM oxidant, for 10 min at 25 °C (50 μl, 25 μM tubulin, 500 μM cys). For repair assays, 1 mM DTT or TCEP was added for an additional 10 min at 22 °C. GTP (1 mM final) was added to induce polymerization and the samples were incubated at 37 °C for 20–25 min. Microtubule polymer was collected by centrifugation at 16,000*g* for 20 min. Control polymerization activity was set at 100% for those samples containing GTP but no oxidant. Controls without GTP were used to establish 0% activity. Supernatant protein concentrations were determined by the BCA protein assay. Protein supernatants were analyzed by SDS–PAGE with Coomassie Blue staining. Protein pellets were dissolved in 6 M guanidine-HCl and the absorbance was measured at 275 nm [1].

CNBr cleavage to detect methionine oxidation

Tubulin (12.5 μM, 250 μM cys, 325 μM methionine) was treated with each oxidant as described for IAF labeling above. Following acidification to pH 2.5 with 70% formic acid, samples were treated with 35–40 mM CNBr O.N. Samples were neutralized to pH 7.4–7.6 with NH_4OH and subjected to SDS–PAGE under reducing conditions on a 7.5% polyacrylamide gel. Proteins were transferred to PVDF, blocked with 3% milk and incubated with mouse anti-β-tubulin (1:2000) for 2 h. The tubulin-antibody complex was visualized using a goat anti-mouse HRP conjugate (1:10000, 1 h) and a chemiluminescent substrate.

Results and discussion

IAF labeling of tubulin cysteines

To assess oxidation of tubulin cysteines, we used the thiol-specific reagent, iodoacetamido-fluorescein (IAF). Because IAF reacts with reduced cysteines only, tubulin labeling will decrease as the dose of oxidant increases. Previous work in our laboratory showed that all 20 cysteines of tubulin, 12 in α- and 8 in β-tubulin, can be labeled and are accessible without denaturants [1]. Fig. 1A shows that labeling of both α- and β-tubulin decreased as the concentration of oxidant, HOSCN or HOCl, increased. This is typical of the oxidants we have studied and no subunit specificity has been observed [3,6,19].

The tubulin preparation used in Figs. 1 and 2 was desalted to remove small molecules present during purification including unbound GTP, glycerol, EGTA and Mg^{2+}. This tubulin was exchanged into PB pH 7.4 because this buffer does not react with oxidants. These methods, including the buffers and ratios of oxidant to tubulin cys are consistent with those we have performed in our studies of tubulin oxidation by $ONOO^-$, NO donors, H_2O_2, HOCl and chloramines [1,3,19,23,24].

In Fig. 1A, tubulin (120 μM cys) was treated with increasing concentrations of HOCl and HOSCN. As the concentration of oxidant increased, labeling with IAF decreased. Neither thiocyanate ion alone nor decomposed HOSCN affected IAF labeling (Fig. 1S). The concentrations of HOCl used were greater than those of HOSCN and yet, more oxidation was observed with HOSCN. To ensure that no oxidant remained, we performed time-course experiments with HOSCN and the reaction was essentially complete after 5 min (Supplemental data Fig. 2S). In the case of 100 μM HOCl treatment,