Acetaldehyde Substoichiometrically Inhibits Bovine Neurotubulin Polymerization

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Abstract

Acetaldehyde is known to form covalent adducts with tubulin and to inhibit microtubule formation. Available evidence indicates that lysine residues are prominently involved in adduct formation. Previous work has shown that lysines on tubulin can be divided into two general classes based upon their reactivity toward acetaldehyde; those of normal reactivity ("bulk" lysines) and a highly reactive lysine (HRL) located on the α-polypeptide subunit. We took advantage of the fact that the HRL is unreactive when tubulin is in the microtubule form to differentiate the effects of bulk from HRL adducts on tubulin polymerization. Under conditions where both bulk lysines and HRL formed adducts, 0.2 mol acetaldehyde/mol tubulin caused complete inhibition of polymerization. When we modified bulk lysines, but not HRL, tubulin polymerized essentially normally. Finally, when we first blocked bulk lysines on microtubules (HRL unreactive) using unlabeled acetaldehyde and then measured the amount of [^{14}C]acetaldehyde adduct formed with tubulin after depolymerization (HRL reactive), 0.08 mol acetaldehyde/mol tubulin resulted in completely impaired polymerization. These data show that microtubule formation is very sensitive to even small mole fractions of acetaldehyde-modified tubulin (especially with HRL) and further suggest that small amounts of acetaldehyde adduct could be damaging to cytoskeleton function in the cell.

Introduction

We have proposed that acetaldehyde, the first metabolite of ethanol, may contribute to alcoholic liver injury by forming stable covalent adducts with hepatocellular proteins (1). This theory is consistent with the central role of the liver in ethanol metabolism, as well as the observation that the liver is a principal site of ethanol-induced damage in the body. It is now well established in several studies from our group and others that acetaldehyde reacts with a variety of pure proteins to form both stable and unstable adducts (2, 3). Further studies have shown that acetaldehyde-protein adduct formation actually occurs in the liver during ethanol oxidation (4). Chemical characterization of reaction products has suggested a prominent role for lysine residues in the acetaldehyde protein interaction (5); however, the exact chemical structures and target proteins of the physiologically relevant in vivo adducts have yet to be fully explained.

Our experimental approach to this area has been to attempt to gain basic information concerning the interaction of acetaldehyde with various model proteins in vitro. Recently we have chosen to concentrate on the cytoskeletal protein, tubulin, for a variety of reasons including its obvious biologic importance and earlier observations that microtubule-dependent processes in the liver seem to be impaired by acetaldehyde (reviewed in reference 6). More recent data from H. Sternlicht's laboratory (Case Western Reserve University, Cleveland, OH) that indicates that there is a special lysine residue located on α-tubulin that is both unusually reactive toward aldehydes and critically important to tubulin polymerization and provides biochemical rationale to investigate the effects of acetaldehyde lysine adducts on tubulin function (7–9). In agreement with Sternlicht and co-workers, we have recently found that lysine residues on tubulin can be divided into two general classes with regard to their reactivity toward acetaldehyde; those of normal reactivity (bulk lysines) and the highly reactive lysine (HRL)[1] located on the α-polypeptide subunit of the tubulin molecule (10). We took advantage of the fact that the HRL is unreactive when tubulin is in the microtubule form to differentiate the effects of HRL from bulk acetaldehyde-tubulin adducts. In this report, we show that relatively small quantities of acetaldehyde-modified tubulin are sufficient to impair the process of microtubule formation and, furthermore, that HRL adducts are more deleterious to tubulin polymerization than adducts in general.

Methods

Materials

Fresh adult beef brains were obtained at the time of slaughter and were rapidly transported to the laboratory on ice. (1,2-[^{14}C])Acetaldehyde (4.9 mCi/mmol) and Aquasol were purchased from New England Nuclear (Boston, MA). Acetaldehyde was received from the manufacturer frozen as an aqueous solution (1 mCi/ml), thawed and diluted to 200 μCi/ml with distilled water, rapidly refrozen, and stored at −80°C. GTP type IIS, EGTA, *N*-morpholinoethane sulfonic acid (MES) and nonradioactive acetaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Glycerol was obtained from Aldrich Chemical Co. (Milwaukee, WI). Electrophoresis equipment and supplies were obtained from Bio-Rad Laboratories (Richmond, CA). All other reagents and supplies were of analytical grade.

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1. *Abbreviations used in this paper:* HRL, highly reactive lysine; MES, *N*-morpholino ethane sulfonic acid.

Protocols

Protein purification. Tubulin was isolated and purified from bovine brain by a modification of the method of Shelanski et al. (11). Upon completion of the Shelanski cycle purification method, the tubulin was subjected to G-25 column chromatography to remove GTP. The tubulin was stored under liquid nitrogen in reassembly buffer consisting of 20 mM MES, 70 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, and 2 M glycerol, pH 6.7. Purity of tubulin and microtubules was confirmed using SDS-PAGE and electron microscopy, respectively.

Our protein preparation typically contained 80–90% tubulin (α:β dimer) with other bands consisting mainly of high-molecular weight microtubule-associated proteins. Protein concentrations were measured by the method of Lowry et al. (12), using BSA as a standard and applying a correction factor of 1.2 for tubulin. The validity of this correction factor was previously verified by using absorbance measurements and known molar extinction coefficients (10).

Acetaldehyde-binding assay. Standard reaction mixtures consisted of cycle-purified tubulin (HRL reactive) or microtubules (HRL unreactive) at a concentration of 3.00 mg/ml. Appropriate dilutions of [^{14}C]acetaldehyde were added to reaction mixtures to achieve the final desired concentration. Incubation time was adjusted to a length necessary for inhibition of polymerization to occur, as confirmed by the change in optical density at 350 nm (13). Reaction vessels were polyethylene and were sealed to minimize the loss of volatile radioactivity. Incubations were carried out at 37°C with constant agitation. Postincubation, the samples were subjected to exhaustive dialysis and stable protein-bound radioactivity was determined as described by Donohue et al. (2).

Gel electrophoresis. Dialyzed reaction mixtures were subjected to SDS-PAGE using the modified Laemmli system (10) and stained with Coomassie blue. Tubulin α and β bands were well resolved using this system. The bands corresponding to the α and β polypeptides of tubulin were cut out and solubilized using 0.2 ml of 30% H_2O_2 at 75°C. After being cooled to room temperature, 0.5 ml of 2% thiourea was added to each sample directly into the scintillation vial. Samples were counted with 15 ml Aquasol after acidification with glacial acetic acid. The relative distribution of [^{14}C]radioactivity between the two polypeptide chains was expressed as a ratio of α to β. As established by Szasz et al. (7), an α/β ratio ~ 1.50 for free tubulin confirmed that the HRL groups were reactive during the assay and participating in adduct formation with acetaldehyde. An α/β ratio of ~ 1.00 confirmed that the HRL groups remained unreactive when tubulin was in the polymerized state (microtubule form).

Tubulin polymerization assay. Polymerization of tubulin was monitored by measuring the increase in turbidity at 350 nm (13), using a spectrophotometer (model DU-70; Beckman Instruments, Fullerton, CA). An increase in turbidity at 350 nm has been shown to be directly proportional to the amount of microtubules formed (14). The polymerization reaction was initiated by warming a solution of depolymerized tubulin to 37°C and adding 0.5 mM GTP (the cofactor for polymerization). Recently we have shown that unstable adducts do not cause inhibition of polymerization (6); therefore, all acetaldehyde-modified tubulin samples were submitted to polymerization assay directly without attempting to remove unstable adducts. Appropriate controls consisted of samples of tubulin that had been incubated and manipulated exactly as had acetaldehyde-modified samples, except that no acetaldehyde was added.

Results

Initial experiments were conducted to establish conditions for modification of tubulin and microtubules by acetaldehyde. In agreement with our previous studies (10), tubulin formed ~ 20% more stable adducts with acetaldehyde when it was in the free (depolymerized) form as compared with the microtubule form (Fig. 1 *A*). When reaction products were submitted to SDS-PAGE to resolve the α and β polypeptides of tubulin/microtubules, the distribution of radioactivity was markedly different in these two situations (Fig. 1 *B*). Under these conditions, free tubulin formed ~ 1.5 times as much stable covalent adduct on the α chain as on the β chain; whereas, microtubules exhibited equal formation of adducts on the two chains. As extensively described by Szasz et al. (7), this effect allowed us to prepare two types of acetaldehyde-modified microtubular protein: (a) modified tubulin with both bulk lysines and HRL lysines modified and (b) modified microtubules with bulk lysines modified but HRL lysine not modified.

We next set out to determine the effects of the various types of acetaldehyde adducts on the ability of tubulin to form microtubules (polymerization assay). First we explored the effects of bulk plus HRL adducts on tubulin polymerization. Samples of tubulin were modified to varying extents with acetaldehyde and submitted to polymerization assay. As shown in Fig. 2,

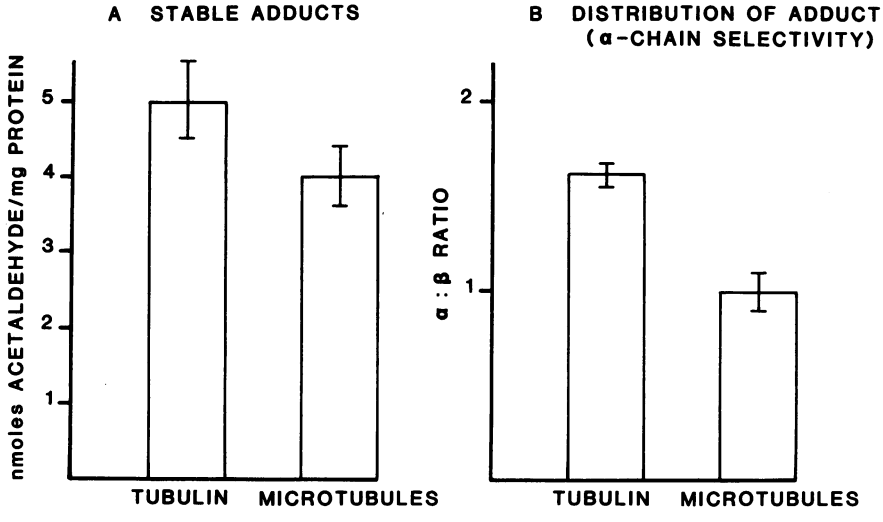

Figure 1. Acetaldehyde adduct formation with cycle-purified neurotubulin and microtubules. Depolymerized tubulin (3.00 mg/ml) was suspended in tubulin storage buffer with the final glycerol concentration adjusted to 0.6 M at pH 6.7. Microtubules were formed by the addition of 0.5 mM GTP to the tubulin solution and incubation at 37°C for 15 min. Tubulin and microtubules were incubated in tightly sealed vessels at 37°C with [^{14}C]acetaldehyde (5 mM). (*A*) After exhaustive dialysis, reaction mixtures were processed for determination of stable protein-bound radioactivity as described in Methods. (*B*) The tubulin and microtubule samples were subjected to SDS-PAGE as described in Methods and the radioactivity associated with the α and β chains was determined. The results are expressed as a ratio and represent the mean±SEM of five determinations.

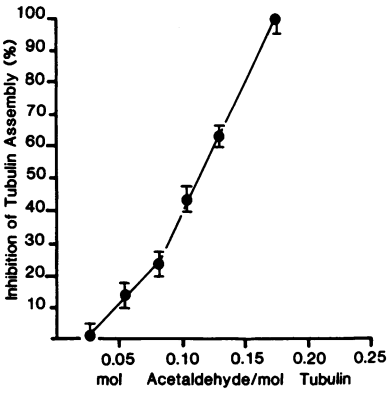

Figure 2. The effects of bulk lysine plus HRL adducts on tubulin polymerization. Tubulin (3.00 mg/ml) was incubated at 37°C with 5 mM [^{14}C]acetaldehyde. At various time points, the mol of [^{14}C]acetaldehyde bound per mol of tubulin was measured and the ability of the modified tubulin to polymerize was determined. Inhibition of tubulin assembly was ascertained by comparing the change in optical density at 350 nm of the adducted tubulin to control samples incubated for an equal amount of time without acetaldehyde. Results represent the mean±SEM of five determinations.

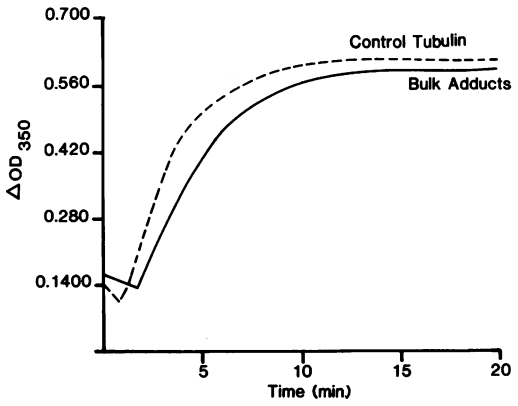

Figure 3. The effect of bulk lysine acetaldehyde adducts on tubulin polymerization. Tubulin (6.00 mg/ml) was incubated with 0.5 mM GTP at 37°C for 15 min; polymerization into microtubules was confirmed by measuring the change in optical density at 350 nm. Upon completion of tubulin polymerization to microtubules, half of the microtubules were incubated with 5 mM acetaldehyde (——) at 37°C for a length of time equal to that necessary to inhibit polymerization of free tubulin. The other half served as a nontreated control (- - -). After incubation, the samples were dialyzed against warm assembly buffer containing 2 mM GTP and 2 M glycerol for 6 h. After dialysis, the acetaldehyde-treated microtubules (bulk adducted) and the control microtubules were placed in separate homogenizing vessels and homogenized at 1,000 rpm for 2 min and placed on ice for 30 min to cause disassembly back to tubulin. The bulk-adducted tubulin and identically treated control tubulin samples were then subjected to the polymerization assay as described in Methods.

there was a correlation between the extent of adduct formation and impaired ability to polymerize. We determined that for complete inhibition of tubulin polymerization, it was necessary to have only 0.20 mol of acetaldehyde bound per mol of tubulin. Further confirmatory experiments were done in which acetaldehyde was reacted with tubulin at different concentrations and temperatures to show that adduct formation, rather than exposure to experimental conditions, was responsible for impaired polymerization. Included in these conditions were prolonged cold incubations (necessary to maintain tubulin stability) in which exposure to concentrations of acetaldehyde as low as 50 μM resulted in sufficient adduct formation to inhibit polymerization. In all cases, there was a correlation between adduct formation and polymerization impairment, independent of reaction conditions (data not shown). Because it was our goal to differentiate the effects of bulk from HRL adducts, we took samples of acetaldehyde-modified microtubules (bulk lysines modified but HRL unreacted), depolymerized them, and then submitted them to the polymerization assay. This preparation of acetaldehyde-modified microtubule protein polymerized essentially normally (Fig. 3), suggesting that bulk acetaldehyde-tubulin adducts have little or no role in the acetaldehyde-induced defect in tubulin polymerization.

As it appeared that the HRL adduct was prominently involved in the acetaldehyde-induced defect in tubulin polymerization, we devised an experimental strategy to demonstrate this directly. We pretreated microtubules with nonradioactive acetaldehyde to block bulk lysines, depolymerized them (exposing the HRL), and then reacted them with [^{14}C]acetaldehyde. We were then able to correlate radioactively labeled HRL (confirmed by α/β ratios) adducts with inhibited tubulin polymerization. As shown in Fig. 4, under these conditions HRL adducts were confirmed to be chiefly responsible for defective polymerization. When bulk lysines were blocked with unlabeled acetaldehyde, only 0.08 mol of [^{14}C]acetaldehyde per mol of HRL was necessary for tubulin polymerization to be completely inhibited.

Discussion

Recent studies in our laboratory have confirmed that acetaldehyde can form covalent adducts with tubulin as described for various other proteins (10). Lysine residues are believed to be

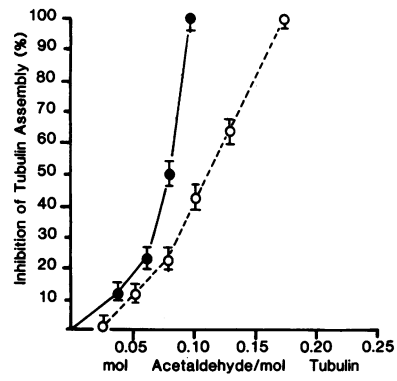

Figure 4. The effects of HRL adducts on tubulin polymerization. Tubulin was polymerized to microtubules by addition of 0.5 mM GTP and incubated at 37°C for 15 min. Polymerization was confirmed by measuring the change in optical density at 350 nm. Microtubules (4.00 mg/ml) were then incubated with 5 mM nonlabeled acetaldehyde at 37°C for a time period necessary for complete inhibition of assembly of an equal amount of free tubulin to occur. Dialysis was carried out against 1 liter of warm assembly buffer with 2 mM GTP and 2 M glycerol. Maintenance of the samples in the microtubule form was confirmed by optical density measurement at various times throughout the dialysis period that exceeded 6 h. The microtubules were then disassembled by homogenization and incubation on ice for 30 min. Adducted tubulin (3.00 mg/ml) (bulk adducts only) was then incubated at 4°C with 5 mM [^{14}C]acetaldehyde. A temperature of 4°C was used to assure that the free GTP did not promote reassembly. At various time points during the incubation, the mole ratio of stable acetaldehyde adducts to tubulin was determined, along with the ability of the HRL-labeled tubulin to polymerize. Inhibition of assembly was determined by comparing the difference in optical density of the HRL-labeled tubulin to that of the control bulk adducted tubulin. The inhibition of polymerization by HRL-adducted tubulin (——) compared with the inhibition observed with total adducted tubulin (HRL and bulk) (- - -) are shown.

the primary amino acid involved in stable acetaldehyde-tubulin adduct formation (5, 10). The results of our initial studies have shown dramatic differences in acetaldehyde binding characteristics between free tubulin and microtubules. Free tubulin bound ~ 20% more acetaldehyde than did microtubules. However, when adduct distribution was determined after electrophoretic resolution of the α- and β- subunits of tubulin, free tubulin formed ~ 1.5 times more stable adduct on its α chain as on its β chain; whereas, microtubules showed an equal distribution of adducts on the two polypeptide chains. These observations confirmed that the α chain of free tubulin, but not microtubules, has an accessible HRL residue as previously reported by Szasz et al. (7) and Jennett et al. (10). Furthermore, additional work from Sternlicht's laboratory has suggested that this HRL group is also extremely important in the proper function of tubulin (8, 9); therefore, we set out to determine if the HRL adduct was responsible for the acetaldehyde-induced defect in tubulin polymerization that we have previously reported (13). Previous studies in our laboratory (15) and others (16) have suggested that stable acetaldehyde adducts may be involved in the acetaldehyde-induced defect in tubulin polymerization. However, these early studies did not adequately address any differential role of HRL versus bulk adducts in this effect.

The initial functional studies were conducted to determine the total amount of adduct (HRL plus bulk) necessary to impair tubulin polymerization. Only 0.2 mol of bound acetaldehyde per mol of tubulin were required for complete impairment of assembly. We determined, by varying incubation time and temperature, along with tubulin and acetaldehyde concentrations, that the critical factor for inhibition of assembly was the amount of stable adduct formed. Because it appeared that relatively small mole fractions of adducted tubulin were sufficient to impair microtubule formation, these observations led us to wonder if specific acetaldehyde-lysine adducts on tubulin were capable of substoichiometrically inhibiting tubulin polymerization. Inhibition of microtubule formation at substoichiometric mole ratios is a characteristic of well-established antimicrotubule agents such as colchicine (17).

Because previous results have shown that the HRL group is inaccessible to acetaldehyde in the polymerized state of tubulin (microtubules), we exploited this phenomenon to differentiate the effects of HRL adducts from those of bulk adducts. Under the experimental conditions used in this study to form acetaldehyde-tubulin adducts, bulk adducts likely consist of modified lysine residues, but in addition the amino terminal amino acids of the α- and β-chains (via their free amino group) could also contribute to the quantity of total bulk adducts (8). When microtubules were incubated with acetaldehyde (HRL unreactive) and then depolymerized back to tubulin and subjected to polymerization assay, only a small decrease in polymerization was observed as compared to control tubulin. These data show that the bulk adducts are not responsible for the inhibition of assembly and strongly suggest that the HRL group is chiefly responsible for the detrimental effects of acetaldehyde on tubulin polymerization.

Finally, we set out to prospectively determine if the HRL adducts are specifically responsible for impairment of tubulin assembly. In order to accomplish this, microtubules were incubated with nonlabeled acetaldehyde to selectively block the bulk lysine groups. The microtubules were then disassembled and incubated with [^{14}C]acetaldehyde. These manipulations gave us a tubulin preparation with nonlabeled bulk adducts and specifically labeled HRL adducts. In this case, only 0.08 mol of [^{14}C]acetaldehyde per mol of tubulin needed to be bound (to HRL) for complete inhibition of assembly to occur. These data strongly suggest that HRL-acetaldehyde adducts can substoichiometrically impair microtubule formation. When this observation is coupled with existing data from competition binding studies showing that α tubulin (via HRL) is a preferential target for adduct formation (10), it is tempting to speculate that sufficient adducts may accumulate on tubulin in cellular systems to impair the function of the microtubule system. Chronic functional perturbation of the cytoskeleton may initiate alcoholic liver injury by deranging important microtubule-dependent cellular processes (6).

To date, acetaldehyde-protein adducts have been reported on an unidentified 37-kD protein in the livers of ethanol-fed rats (18). It is important to point out, however, that this adduct was detected in this study by using antisera to reduced acetaldehyde-protein adducts (*N*-ethyl lysine residues) that may not be the physiologically relevant acetaldehyde-protein adduct (19). At present, it is not known whether acetaldehyde adducts actually form on tubulin during hepatic ethanol oxidation. In preliminary experiments, we have detected stable labeling of α tubulin after addition of [^{14}C]acetaldehyde to high-speed hepatic supernates (unpublished). Recent extensive studies have also confirmed that rat liver tubulin shows selective α-chain acetaldehyde binding properties very similar to those of beef brain tubulin (20). Work in progress, aimed at development of highly specific MAbs to nonreduced acetaldehyde-tubulin adducts, will be necessary to determine whether sufficient acetaldehyde-tubulin adducts actually form during ethanol oxidation in the liver to cause cytoskeletal dysfunction.

In summary, we have shown that acetaldehyde-tubulin adducts cause inhibition of tubulin polymerization. Further studies show that α-chain adducts involving an unusually reactive lysine are prominently involved in this effect. Because we have already established that the α chain of tubulin is a preferential site of acetaldehyde adduct formation, these findings raise the possibility that hepatocyte microtubule dysfunction induced by HRL adducts could contribute to alcoholic liver damage. In view of the small mole fractions of acetaldehyde-modified tubulin necessary to inhibit microtubule formation, even small amounts of adduct could be very damaging to cytoskeletal function in cellular systems.

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