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Ethanol impairs microtubule formation via interactions at a microtubule associated protein-sensitive site

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Abstract

Prolonged ethanol abuse has been associated with brain injury caused by impaired synaptogenesis, cellular migration, neurogenesis, and cell signaling, all of which require proper microtubule functioning. However, the means by which ethanol may impair microtubule formation or function and the role that microtubule-associated proteins (MAPs) have in mediating such effects are not clear. In the present studies, purified MAP-deficient (2 mg/mL) and MAP-rich (pre-conjugated; 1 mg/mL) bovine α/β tubulin dimer were allowed to polymerize at 37 °C, forming microtubules in the presence or absence of ethanol (25–500 mM). Microtubule formation was assessed in a 96-well format using a turbidity assay, with absorption measured at 340 nm for 45 min. Additional studies co-exposed α/β tubulin dimers to 50 mM ethanol and purified MAPs (0.1 mg/mL) for 45 min. Polymerization of MAP-deficient tubulin was significantly decreased (at 15–45 min of polymerization) during exposure to ethanol (> 25 mM). In contrast, ethanol exposure did not alter polymerization of α/β tubulin dimers pre-conjugated to MAPs, at any concentration. Concurrent exposure of MAP-deficient tubulin with purified MAPs and ethanol resulted in significant and time-dependent decreases in tubulin polymerization, with recovery from inhibition at later time points. The present results suggest that ethanol disrupts MAP-independent microtubule formation and MAP-dependent microtubule formation via direct actions at a MAP-sensitive microtubule residue, indicating that disruption of neuronal microtubule formation and function may contribute to the neurodegenerative effects of binge-like ethanol intake.

Keywords

tubulin; polymerization; alcoholism; neuronal injury

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Introduction

Brain injury and neurological impairment are consequences of prolonged ethanol exposure in both the developing and adult brain. Deficits in brain volume of cortical and subcortical structures have been noted following prolonged fetal, adolescent, or adult exposure to high doses of ethanol (Archibald et al., 2001; Beresford et al., 2006; Cardenas et al., 2005; Chanraud et al., 2007; Kril et al., 1997; 2008; Meyerhoff et al., 2005; Roebuck et al., 1998; Sowell et al., 2001). Furthermore, losses in brain volume associated with chronic ethanol exposure have also been correlated with deficits in neurocognitive performance in the adult and developing brain (Chanraud et al., 2007; Mattson et al., 2001). The means by which ethanol exposure produces neuronal injury are clearly complex and include activation of several injurious signaling pathways such as those dependent on reactive oxygen species formation, release of pro-inflammatory cytokines and chemokines, and, during ethanol withdrawal, the induction of excitotoxicity (Beresford et al., 2006; Carderas et al., 2005; Cederbaum, 2006; Davidson et al., 1995; Gonzalez, 2005; Meyerhoff et al., 2005; Prendergast et al., 2004; Vallés et al., 2004).

Interactions of ethanol and/or its metabolites with microtubules have also been noted and proposed as a possible means of initiating cell apoptosis in the periphery and central nervous systems (CNS; Ahluwalia et al., 2000; Banan et al., 1998; Bhalla, 2003; Evrard et al., 2006; Gendron et al., 2008; Kannarkat et al., 2006; Lang et al., 1997; Yoon et al., 1998). Some evidence suggests that low, but not high, concentrations of ethanol may increase the phosphorylation of microtubule-associated proteins (MAPs; Ahluwalia et al., 2000). In the periphery, ethanol exposure decreased levels of polymerized tubulin *in vitro*. However, exposure to anti-oxidant compounds attenuated ethanol's effects on tubulin polymerization (Banan et al., 1999), suggesting indirect effects of ethanol on tubulin polymerization associated with ethanol metabolism and formation of reactive oxygen species. Furthermore, hepatocytes from ethanol-fed rats displayed increased assembly of incompetent tubulin, which corresponds to an inability for microtubules to reassemble (Banan et al., 1998). Cultured hepatocytes also displayed increases in acetylated α-tubulin following ethanol exposure, which increases microtubule stability or rigidity and disrupts tubulin depolymerization (Kannarkat et al., 2006). However, it is not clear if ethanol may directly interact with MAPs or microtubules. Disruption of tubulin polymerization or depolymerization is potentially cytotoxic as either may result in an upregulation of pro-apoptotic Bcl-2 proteins, release of cytochrome c from mitochondria, and activation of apoptotic signaling (Bhalla, 2003; Giacca, 2005).

In the CNS, ethanol exposure also results in microtubule dysfunction. Following chronic ethanol exposure (4 months), a significant decrease in the density of microtubules in the dentate gyrus of the hippocampus was observed, though significant recovery of microtubule density was observed during withdrawal from ethanol intake (Lang et al., 1997). The production of ethanol's metabolite, acetaldehyde, was interpreted to contribute to this effect. Acetaldehyde, which may be produced at low concentrations in the brain via interactions with catalase or microsomal ethanol oxidizing systems, irreversibly binds to α-tubulin, thus inhibiting tubulin polymerization (Tuma et al., 1991). MAP density also appears to be

affected by acute exposure to low concentrations (6–48 mM) of ethanol. Exposure to ethanol results in phosphorylation of MAP-2, a MAP primarily located in the cell body and dendrites, via induction of cAMP-independent protein kinase (Ahluwalia et al., 2000). Phosphorylation of MAP-2 has been shown to inactivate the protein, disrupting MAP-promoted microtubule assembly (Ahluwalia et al., 2000). Furthermore, chronic exposure to ethanol has been shown to significantly decrease MAP-2 immunoreactivity, in the CA1 region of the hippocampus, the striatum, and the frontal cortex of rodents (Evrard et al., 2006). Exposure to a high concentration of ethanol (100 mM) has also been suggested to delay tau clearance, a MAP found primarily in axons, resulting in reduced cell viability (Gendron et al., 2008).

While evidence clearly exists that ethanol exposure may alter microtubule dynamics or MAP function/density, it is not known if ethanol directly interacts with tubulin, microtubules, and/or microtubule-associated proteins to produce such detrimental effects. The present *ex vivo* studies employed purified MAP-deficient and MAP-rich bovine tubulin to examine effects of ethanol exposure on MAP-independent and MAP-dependent microtubule formation.

Materials and methods

Preparation of MAP-deficient and MAP-rich tubulin

Purified (< 3% MAPs) bovine tubulin (2 mg/mL; MAP-deficient tubulin) and MAP-rich bovine tubulin (MAPs pre-conjugated to tubulin; > 99% pure) (1 mg/mL) were reconstituted in tubulin polymerization buffer (G-PEM, 80 mM piperazine-N,N′-bis(2-ethanesulfonic acid) sequisodium salt, 2.0 mM $MgCl_2$, and 0.5 mM ethylene glycol-bis(*b*-amino-ethyl ether)-*N,N,N′,N′*-tetra-acetic acid (Cytoskeleton Inc., Denver, CO, USA). Ethanol (0–500 mM) was diluted into G-PEM and then aliquotted (50 μL) into a pre-warmed (37°) 96-well plate. Lower mM concentrations (0, 25, 50, or 75 mM) of ethanol were chosen to reflect binge-relevant blood alcohol concentrations (Bedford et al., 2006; Caudill et al., 2006; Maier & West, 2001). Higher mM concentrations of ethanol (100–500 mM) were chosen to determine the potential concentration-dependence of ethanol's effects on polymerization of MAP-rich tubulin. Immediately following the reconstitution of the tubulin, samples were added to either aliquotted ethanol buffer or control buffer (G-PEM; 50 μL; total volume 100 μL). A kinetic assay was conducted in a Beckman-Coulter DTX 880 Multimodal Detector, with absorbance at 340 nm measured every 5 min for 45 min (Beckman-Coulter, Fullerton, CA, USA). All tubulin assays were conducted in triplicate.

Additional studies were conducted employing MAP-deficient tubulin that was incubated with purified MAP protein fraction to examine acute effects of MAP and ethanol on microtubule formation. MAP protein fraction (0.1 mg/mL; Cytoskeleton, Inc.) and bovine tubulin (2 mg/mL) were reconstituted in G-PEM buffer. A variety of MAP protein fraction concentrations (0.001–0.5 mg/mL) was initially examined for stability. However, the 0.1 mg/mL concentration of MAP protein was chosen for ethanol tubulin polymerization experiments because it produces a robust and time-dependent enhancement of microtubule formation. Ethanol (50 mM) was also diluted in G-PEM buffer and added to reconstituted bovine tubulin (with or without the addition of MAPs). Bovine tubulin with or without

ethanol (50 μL) and MAP protein fraction (50 μL) was diluted in G-PEM and placed into a pre-warmed 96-well plate. The assay was conducted in a Beckman-Coulter DTX 880 Multimodal Detector, with absorbance at 340 nm measured every 5 min for 45 min.

Statistical analyses

To analyze effects of ethanol exposure on microtubule formation *ex vivo*, two-way repeated measures analyses of variance (ANOVA; treatment × time) were conducted (SigmaStat, Systat Software, San Jose, CA). *Post hoc* comparisons were made using the Holm-Sidak method.

Results

Polymerization of MAP-deficient tubulin

Spontaneous polymerization of tubulin dimers into microtubules was observed at each time point of observation with maximal polymerization achieved after 35 min of incubation (Fig. 1). Ethanol exposure significantly inhibited polymerization of MAP-deficient tubulin dimers into microtubules with complete suppression of polymerization by ethanol concentrations of 50 and 75 mM (treatment × time: ($F_{[64,368]} = 76.144$, $p < 0.001$). Exposure to each concentration of ethanol reduced polymerization of MAP-deficient tubulin, though significant decreases were not observed until 10 (50 and 75 mM) and 15 min (25 mM; Fig. 1) after the start of ethanol exposure. Inhibition of tubulin polymerization was significantly greater for 50 and 75 mM ethanol, as compared to 25 mM ethanol, at several later times of polymerization. Thus, particularly at the early time points of observation, such as 10 min, ethanol suppression of microtubule formation was concentration dependent.

Polymerization of MAP-rich tubulin

Polymerization of MAP-rich tubulin, in the absence of ethanol exposure, was significantly greater than polymerization of MAP-deficient tubulin, regardless of treatment or time (treatment: ($F_{[3,242]} = 82.322$, $p < 0.001$; time: ($F_{[3,242]} = 527.164$, $p < 0.001$) (Fig. 2). At each time of observation, polymerization of MAP-rich tubulin was nearly 2-fold greater than that of MAP-deficient tubulin. However, polymerization of MAP-rich tubulin was not affected by co-exposure to ethanol at any time or concentration of ethanol (Fig. 3A). Additional studies examined the effects of higher concentrations of ethanol (100 or 500 mM) on microtubule formation and also failed to demonstrate any effect of ethanol exposure on polymerization of MAP-rich tubulin (Fig. 3B).

MAP-promoted tubulin polymerization

A final series of studies co-exposed MAP-deficient tubulin to both ethanol (50 mM) and purified, exogenous MAPs (0.1 mg/mL) for the duration of the 45 min assay. Addition of purified bovine MAPs to α/β tubulin dimer solution significantly increased the absolute level of tubulin polymerization, at each time of observation (Fig. 4), as compared to polymerization of MAP-deficient tubulin. However, the addition of ethanol (50 mM) significantly inhibited MAP-promoted tubulin polymerization at early time points of observation. This inhibition produced polymerization levels nearly identical to those observed with MAP-deficient tubulin. Further, a significant interaction between treatment

and time was observed (treatment × time: ($F_{[40,590]} = 2.673$, $p < 0.001$). Exposure to ethanol (50 mM) resulted in a decrease (~30%) of MAP10-promoted tubulin polymerization during the first 20 min of the assay. During the final 25 min of the assay, however, no significant differences between MAP-promoted tubulin polymerization were observed, suggesting a reversible, competitive effect of ethanol on MAP-dependent microtubule formation.

Discussion

Cellular division, movement of organelles, structural support of the cell as the cytoskeleton, and movement of vesicles and intracellular proteins are accomplished via the formation and function of microtubules (Bhalla, 2003; Inoué & Salmon, 1995; Karsenti & Vernos, 2001; Nogales & Wang, 2006; Tassin & Bornens, 1999). Microtubules are polymers comprised of protofilaments consisting of polymerized α and β tubulin dimers (Bhalla, 2003). To further stabilize microtubule polymerization, microtubule-associated proteins (MAPs) regulate microtubule lattice formation, as well as microtubule dynamics (Galbraith & Gallant, 2000; Plassart-Schiess et al., 2001). The current studies examined the effects of ethanol exposure on microtubule formation, in the presence or absence of purified microtubule-associated proteins (MAPs). Results of these studies demonstrate that acute ethanol exposure, even in the likely absence of significant metabolism to acetaldehyde, can directly inhibit MAP-independent and MAP-promoted microtubule formation, even at concentrations as low as 25 mM. Previous work has suggested that acetaldehyde production may be necessary for the loss of microtubule density in hippocampi of ethanol-fed mice (Lang et al., 1997). However, that conclusion is not clearly supported by the present findings. It is critical to note, however, that polymerization of α/β tubulin dimers pre-conjugated to bovine MAPs was not altered by exposure to any concentration of ethanol (< 500 mM). Polymerization of α/β tubulin dimers in the presence of MAPs was approximately 2-fold greater than that of MAP-deficient tubulin, demonstrating the facilitatory role of MAPs in promoting microtubule stability. The resistance of microtubule formation to disruption by ethanol, even at heroic concentrations, suggests a specific effect of ethanol that is clearly not associated with proteolytic actions. Thus, the effect of ethanol was specific to at least 2 distinct molecular interactions with α/β tubulin dimers, one MAP-independent (Fig. 1) and one that was MAP-dependent (Fig. 4).

Evidence of an interaction between ethanol and MAPs is also seen in data demonstrating that concurrent exposure to a binge-like ethanol concentration with exogenous purified MAPs significantly reduced microtubule formation. This suggests that ethanol interferes with the facilitatory role that MAPs have in promoting microtubule formation and function, possibly in a competitive manner. However, there was recovery of MAP-promoted microtubule formation using this preparation. This recovery of MAP-promoted tubulin polymerization may be due to ethanol evaporation, although this is unlikely since disruption of the polymerization of MAP-deficient tubulin persisted throughout the assay, without recovery. Regardless, the present findings clearly indicate that ethanol exposure, at binge-relevant concentrations, may significantly attenuate microtubule formation in the CNS via multiple mechanisms.

All MAPs, such as MAP-2 and Lis-1 among others, contain two binding domains, one which binds to the microtubule surface, while the other faces outward (Chen et al., 1992). The microtubule-binding domain allows for greater nucleation of unpolymerized tubulin, through stabilization of small tubulin oligomers, thus promoting microtubule formation and stability (Chen et al., 1992). Impairment of MAP action produces dysfunction of tubulin polymerization and instability of microtubules. Disruption of proper microtubule functioning could have deleterious effects on cellular functioning and survival, both in the developing and mature organism. Inhibition of microtubule formation has significant implications for mitosis, as well as intracellular trafficking, likely contributing to mitotic arrest at the metaphase/anaphase transition, formation of the mitochondrial permeability transition pore, and the subsequent initiation of apoptotic signaling involving the upregulation of pro-apoptotic Bcl-2 family proteins such as Bax/Bad/Bim and subsequent caspase activation (Bhalla, 2003; Giacca, 2005). This is likely a critical aspect of both hepatic injury associated with chronic ethanol exposure (e.g., Kondili et al., 2005) and ethanol-associated abnormalities in fetal brain and craniofacial development (e.g. Sulik et al., 1988). Thus, the present studies extend our understanding of ethanol's effects on microtubule dynamics and the resulting cytotoxic outcomes that likely occur in many distinct cell types. We have previously demonstrated that disruption of microtubule formation *in vitro* is associated with the toxicity of organophosphate pesticide exposure and exposure to the human immunodeficiency virus-1 transcription regulator Tat (Butler et al., 2011; Prendergast et al., 2007). Thus, these and the current findings suggest that disruption of microtubule formation may well be a unitary mechanism underlying the cytotoxic effects of several biological and environmental toxins.

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Abbreviations

MAPS	microtubule-associated proteins
MAP-2	microtubule-associated protein 2
cAMP	cyclic adenosine monophosphate
G-PEM	piperazine-N, N′-bis(2-ethanesulfonic acid) sequisodium salt
ANOVA	analysis of variance
CNS	central nervous system

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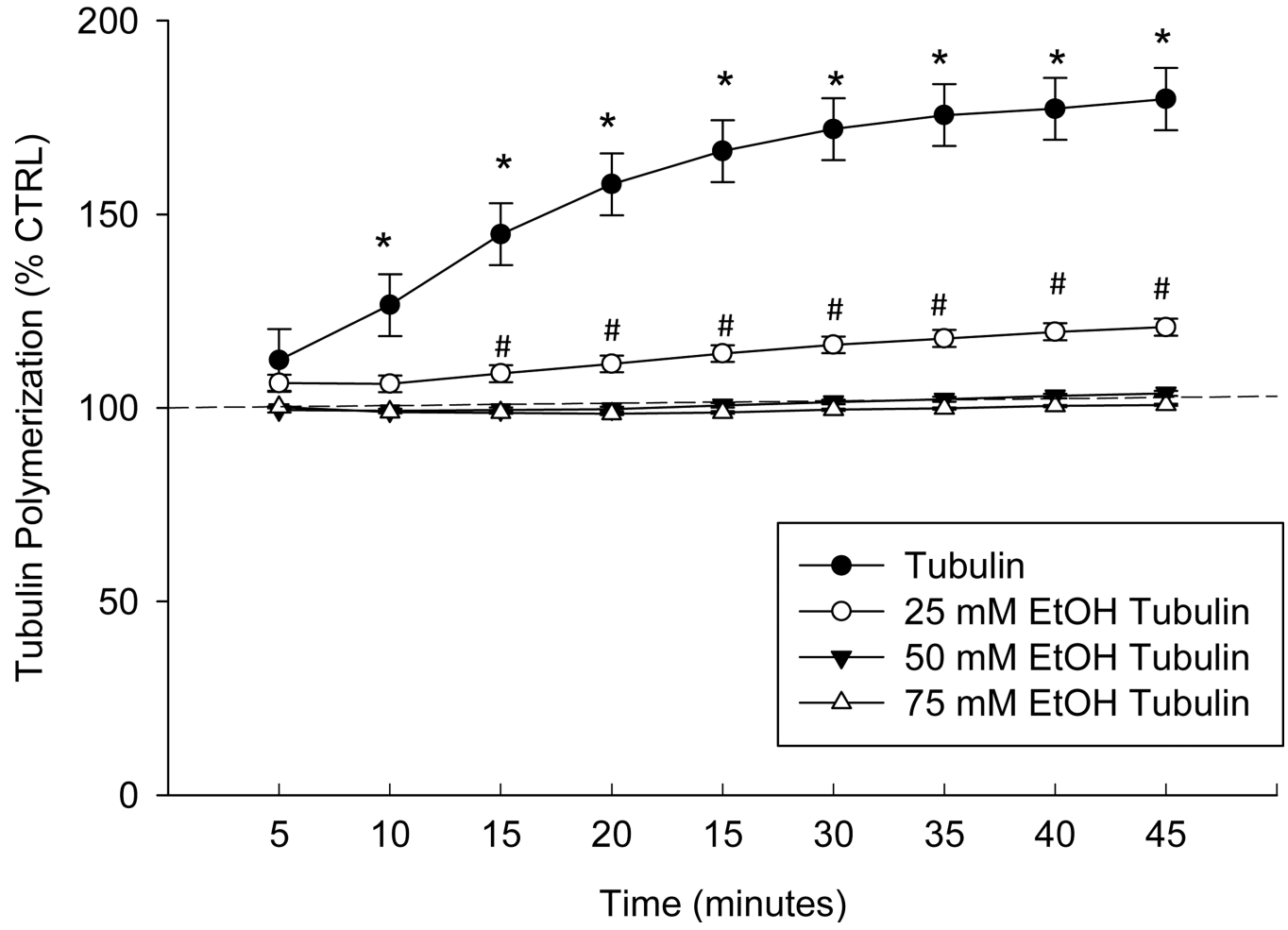

Figure 1.

Polymerization of MAP-deficient tubulin was significantly inhibited by exposure to each concentration of ethanol (25, 50 and 75 mM), at each time point of observation. Concentration-dependent effects were observed as microtubule formation was entirely inhibited by co-exposure to 50–75 mM ethanol, but only partially inhibited by exposure to 25 mM ethanol. *= $p < 0.05$ vs. control; #= $p < 0.05$ vs. 50 and 75 mM ethanol.

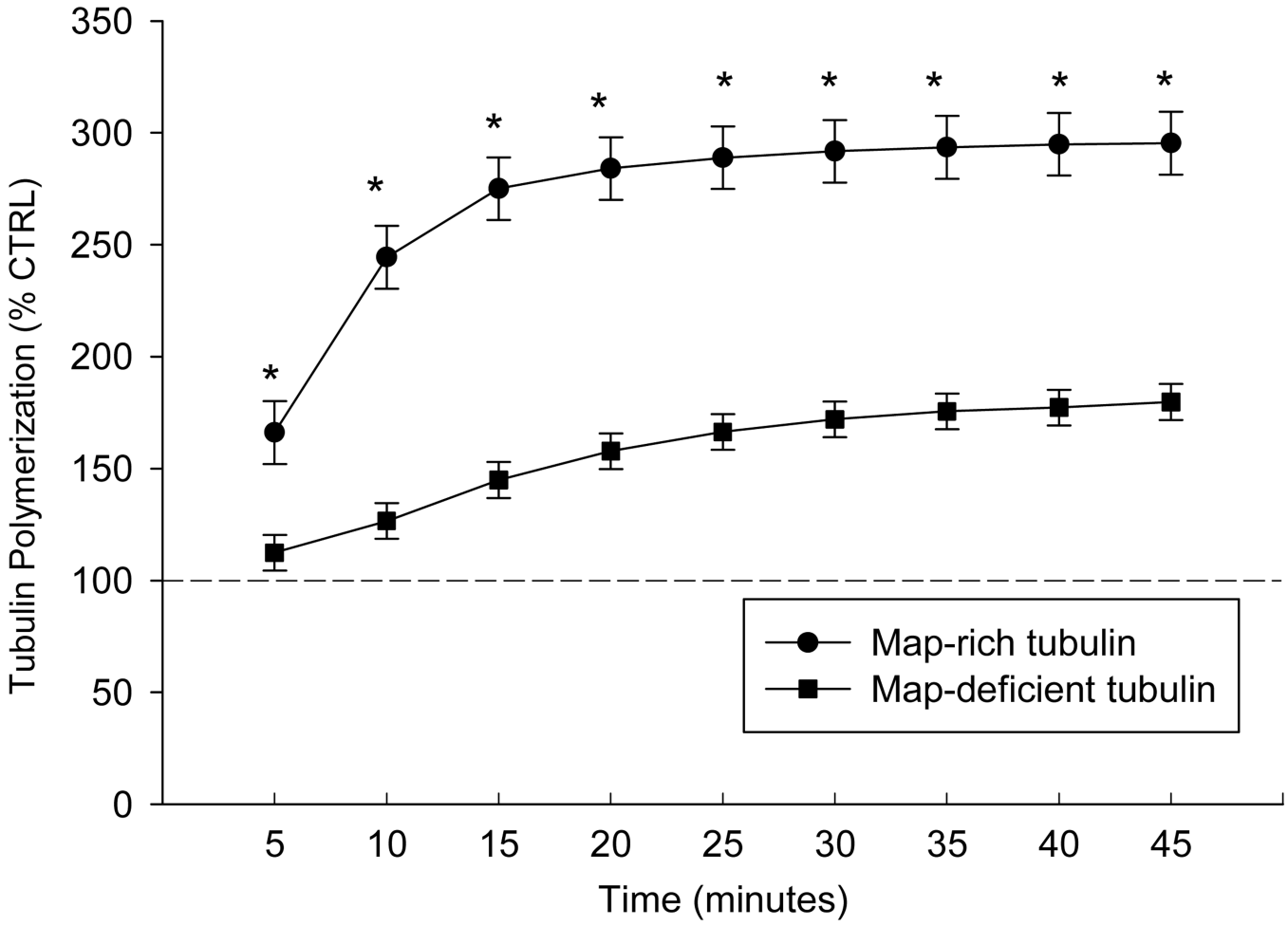

Figure 2.

α/β tubulin dimers pre-conjugated to MAPs polymerized at a rate nearly 2-fold greater than did MAP-deficient tubulin. *= $p < 0.05$ vs. MAP-deficient tubulin.

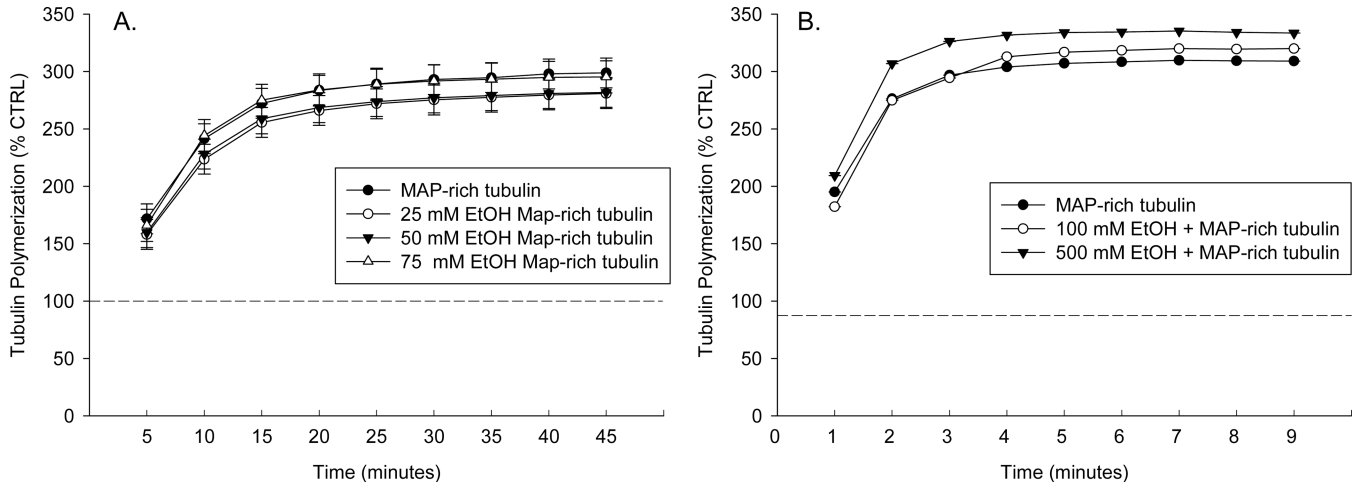

Figure 3.

Co-exposure of α/β tubulin dimers with ethanol (25–500 mM) did not reduce polymerization of MAP-conjugated tubulin at any time point.

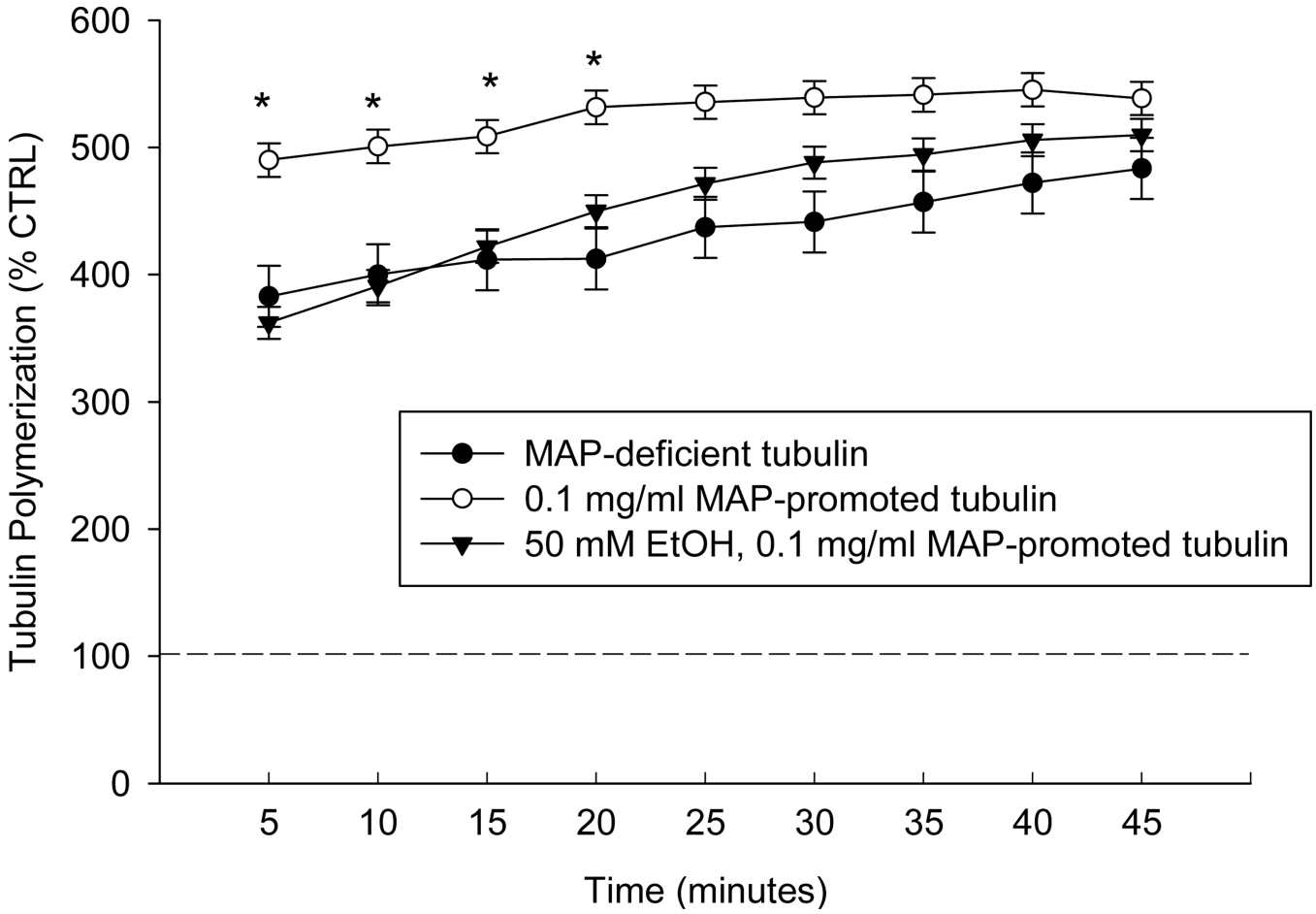

Figure 4.

Addition of purified MAPs to tubulin polymerization buffer produced significant increases in absolute α/β tubulin dimers polymerization, as compared to that of MAP-deficient α/β tubulin dimers. Exposure to 50 mM ethanol significantly decreased MAP-promoted tubulin polymerization during the first 20 min of exposure, but polymerization of α/β tubulin dimers exposed to ethanol was similar to that of ethanol-naïve samples at later time points of measurement. *= $p < 0.05$ vs. MAP-deficient tubulin and ethanol-exposed samples.