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Research report

Mechanisms of 4-hydroxynonenal-induced neuronal microtubule dysfunction

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Abstract

We have previously demonstrated that neuronal microtubules are exquisitely sensitive to the lipid peroxidation product 4-hydroxynonenal (HNE). The mechanism, however, by which HNE disrupts the microtubules, is not known. Sulfhydryl groups of protein-cysteines constitute main targets of HNE. Indeed, HNE is mainly detoxified by conjugation to glutathione (GSH), a reaction that leads to depletion of cellular GSH. GSH maintains protein sulfhydryl groups in the reduced form and has been implicated in the regulation of cytoskeletal function. Here, we assess what role depletion of cellular GSH plays in the HNE-induced microtubule disruption. We demonstrate that HNE and its intracellularly activated tri-ester analog, HNE(Ac)$_3$, cause substantial GSH depletion in Neuro2A cells. However, other compounds inducing GSH depletion had no effect on the microtubule network. Therefore, HNE-induced depletion of cellular GSH does not contribute to the HNE-induced microtubule disruption. We previously demonstrated that another main cellular target of HNE is tubulin, the core protein of microtubules containing abundant cysteines. The functional relevance of this adduction, however, had not been evaluated. Here, we demonstrate that exposure of Neuro 2A cells to HNE or HNE(Ac)$_3$ results in the inhibition of cytosolic taxol-induced tubulin polymerization. These and our previous observations strongly support the hypothesis that HNE-adduction to tubulin is the primary mechanism involved in the HNE-induced loss of the highly dynamic neuronal microtubule network.
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1. Introduction

Oxidative stress, and lipid peroxidation in particular, has been implicated in the pathogenesis of several neurodegenerative diseases including Alzheimer's disease [50,68]. The brain with its tremendous oxygen consumption, high levels of lipid, and enrichment of polyunsaturated fatty acids is particularly prone to lipid peroxidation [13,71]. Oxidation of polyunsaturated fatty acids yields reactive aldehydic products such as 4-hydroxynonenal (HNE).

These reactive aldehydes may contribute to neurodegeneration by a number of mechanisms [58,65]. We have previously demonstrated that the neuronal microtubule network is exquisitely sensitive to HNE. We observed microtubule disruption in Neuro2A cells after exposure times as short as 15 min and HNE concentrations as low as 5 μM [56,57]. The mechanism, however, by which this

Abbreviations: BSO, buthione sulfoximine; DEM, diethyl maleate; DMEM, Dulbecco's Modified Eagle Medium; EGTA, ethylenglycol-bis(2-aminoethylether)-*N*,*N*,*N*″,*N*′-tetraacetic acid; FBS, fetal bovine serum; GSH, glutathione; GTP, guanosine 5′-triphosphate; HNE, 4-hydroxy-2(*E*)-nonenal; HNE(Ac)$_3$, 1,1,4-Tris(acetyloxy)-2(*E*)-nonene; HRP, horseradish peroxidase; MAPs, microtubule-associated proteins; MES, 2-[*N*-morpholino]ethanesulfonic acid; PBS, phosphate-buffered saline; SH, sulfhydryl

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aldehyde causes microtubule disruption is not clear. The immediacy of the effects of HNE suggests that this aldehyde either acts directly on the microtubules or alters a factor that regulates microtubule polymerization.

Microtubules are one of three filamentous components of the cytoskeleton. They are decorated with a multitude of microtubule-associated proteins (MAPs) that regulate the dynamics of this cytoskeletal element [27]. The walls of these tubules arise from the polymerization of tubulin, the core protein of microtubules. Tubulin has a high content of cysteines [10,47,61] of which two are crucially important for its polymerization into microtubules [28,37,39,40,41,53,60].

SH-groups of cysteine constitute a main protein-associated target of HNE [16]. Indeed, cellular detoxification of HNE is mainly achieved through conjugation to the cysteine of GSH. This reaction is catalyzed by GSH *S*-transferase and leads to a transient decrease in cellular GSH [12,25,30,33,35,75]. Cellular GSH plays an important role in maintaining protein SH in the reduced form [14] and the cellular SH-status in turn has been implicated in maintaining cytoskeletal organization and function [37,53,55].

Therefore, HNE could either cause microtubule disruption by reducing cellular GSH levels and thus the SH-status of tubulin, or by directly reacting with SH-groups of tubulin or a microtubule regulatory protein. In a first set of experiments, we tested the hypothesis that aldehyde-induced GSH depletion causes the observed microtubule disruption in Neuro 2A cells. In a second set of experiments, we examined the possibility that HNE-adduction to tubulin is the cause for the HNE-induced disruption of the microtubular network.

2. Materials and methods

2.1. Materials

Chemicals required for the synthesis of 4-hydroxy-2(*E*)-nonenal (HNE) and 1,1,4-Tris(acetyloxy)-2(*E*)-nonene (HNE[Ac]$_3$) were purchased from Aldrich (Milwaukee, WI). HNE was synthesized as described [2,18] and stock solutions in either dimethyl sulfoxide or ethanol were kept no longer than one week at −20 °C. The synthesis of 1,1,4-Tris(acetyloxy)-2(*E*)-nonene (HNE[Ac]$_3$) was performed as described [56]. Unless otherwise indicated, materials used for cell culture were from Invitrogen (Grand Island, NY) and all other chemicals were from Sigma (St. Louis, MO).

2.2. Cell culture

Neuro 2A neuroblastoma cells were purchased from American Type Culture Collection (Rockville, MD). For propagation, the cells were seeded at 20 × 10^4 cells/ml in growth medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (1:1) (DMEM/F12) containing 10% fetal bovine serum and penicillin–streptomycin at 100 units/ml and 100 μg/ml, respectively, and subcultured twice weekly. Unless indicated otherwise, before all experiments, the cells were subcultured at the desired cell density and incubated overnight in growth medium. The next morning, the cells were washed with DMEM/F12 three times and incubated in serum-free N2-medium (DMEM/F12 containing penicillin/streptomycin and N2 supplement) for 24 h.

2.3. Glutathione assay

GSH levels were determined as described previously using monochlorobimane (Molecular Probes, Eugene, OR) [26,31,70]. This non-fluorescent cell membrane permeable dye forms a fluorescent adduct with GSH in a reaction catalyzed by glutathione *S*-transferase [67]. The specificity of monochlorobimane for GSH results from the fact that it is conjugated to GSH by glutathione *S*-transferase and has low nonenzymatic reactivity toward GSH and other thiols [66,67]. Comparisons of cellular GSH levels determined by the monochlorobimane assay and by other assays show excellent correlation [6,70]. The fact that we observed near complete signal depletion when cells were incubated with BSO (buthionine sulfoximine), a specific and irreversible inhibitor of γ-glutamyl-cysteine synthetase (an enzyme crucial for GSH biosynthesis), confirms the specificity of this assay in our system.

Neuro 2A cells were plated in round bottom 96-well plates (Sarstedt, Newtown, NC) at 65 × 10^4 cells/ml in growth medium the night before the experiment. After incubation with HNE or HNE(Ac)$_3$, the cells were incubated with 40 μM monochlorobimane in Hank's Balanced Salt Solution (Invitrogen, Grand Island, NY) for 15 min in the incubator. The fluorescence was quantified with a fluorescence microplate reader (Packard, Fluorocount). Data were analyzed by one-way ANOVA followed by repeated *t* tests with Bonferroni correction for multiple comparisons.

2.4. Glutathione *S*-transferase (GST) activity assay

The monochlorobimane GSH assay depends on the enzyme glutathione *S*-transferase. We therefore ensured that HNE does not affect the activity of this enzyme. We quantified GSH *S*-transferase activity in cell lysates of control and HNE-exposed Neuro 2A cells by measuring the increase in A_{340nm} caused by GSH *S*-transferase—catalyzed conjugation of GSH to 1-chloro-2,4-dinitrobenzene [73]. The reaction mixture, 1 ml total volume, contained 1 mM 1-chloro-2,4-dinitrobenzene (λmax = 340 nm, ε = 9600 M^{-1} cm^{-1}, 100× stock in ethanol) and 50 μl cell homogenate in 0.1 M potassium phosphate, pH 6.5. The GSH *S*-transferase activity was the same in lysates of control and HNE treated Neuro 2A cells.

2.5. Immunocytochemistry

For immunocytochemical analysis, 150 μl of Neuro 2A cells at 5 × 10^4 cell/ml were plated onto 35-mm glass bottom

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