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Original Contribution

Stabilization of superoxide dismutase by acetyl-l-carnitine in human brain endothelium during alcohol exposure: Novel protective approach

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ABSTRACT

Oxidative damage of the endothelium disrupts the integrity of the blood–brain barrier (BBB). We have shown before that alcohol exposure increases the levels of reactive oxygen species (ROS; superoxide and hydroxyl radical) and nitric oxide (NO) in brain endothelial cells by activating NADPH oxidase and inducible nitric oxide synthase. We hypothesize that impairment of antioxidant systems, such as a reduction in catalase and superoxide dismutase (SOD) activity, by ethanol exposure may elevate the levels of ROS/NO in endothelium, resulting in BBB damage. This study examines whether stabilization of antioxidant enzyme activity results in suppression of ROS levels by anti-inflammatory agents. To address this idea, we determined the effects of ethanol on the kinetic profile of SOD and catalase activity and ROS/NO generation in primary human brain endothelial cells (hBECs). We observed an enhanced production of ROS and NO levels due to the metabolism of ethanol in hBECs. Similar increases were found after exposure of hBECs to acetaldehyde, the major metabolite of ethanol. Ethanol simultaneously augmented ROS generation and the activity of antioxidative enzymes. SOD activity was increased for a much longer period of time than catalase activity. A decline in SOD activity and protein levels preceded elevation of oxidant levels. SOD stabilization by the antioxidant and mitochondria-protecting agent acetyl-L-carnitine (ALC) and the anti-inflammatory agent rosiglitazone suppressed ROS levels, with a marginal increase in NO levels. Mitochondrial membrane protein damage and decreased membrane potential after ethanol exposure indicated mitochondrial injury. These changes were prevented by ALC. Our findings suggest the counteracting mechanisms of oxidants and antioxidants during alcohol-induced oxidative stress at the BBB. The presence of enzymatic stabilizers favors the ROS-neutralizing antioxidant redox of the BBB, suggesting an underlying protective mechanism of NO for brain vascular tone and vasodilation.

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A tight monolayer of endothelial cells forming the blood–brain barrier (BBB) separates the brain from blood. It is now widely accepted that BBB disruption plays a critical role in neuroinflammation. The cerebral vasculature is vulnerable to oxidative damage caused by noxious agents derived from blood or produced because of primary brain injury. The brain vasculature is the target of oxidants produced by circulating immune cells, by the endothelium itself, and by glial cells during pathologic conditions. Oxidative damage of the brain endothelium is a key event in the pathogenesis of vascular and neurological diseases. Our previous work demonstrated that ethanol (EtOH) is a potent inducer of oxidative and nitrosative damage in brain cells [1–4]. We have delineated the molecular mechanisms of alcohol-induced oxidative damage of the brain endothelium [3,5,6] leading to BBB disruption, enhanced barrier permeability to toxic agents, and immune cell infiltration into the brain [1,3,7]. Alcohol-induced oxidative injury in brain cells occurs via the metabolism of ethanol by alcohol dehydrogenase (ADH) and cytochrome P450-2E1 (CYP2E1) present in all brain cell types, including brain endothelial cells [2,3,6]. The underlying mechanism is that an ethanol metabolite (acetaldehyde; Ach) activates the free radical-generating NADPH oxidase (NOX) and inducible nitric oxide synthase (iNOS), with subsequent production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) causing oxidative/nitrosative damage [1,3,4,8]. Our recent findings indicated that chronic alcohol exposure not only increases oxidative damage but also enhances adhesion of immune cells and their infiltration into the brain [8].

The redox state of oxidants and antioxidants is such that alcohol-induced oxidative stress is counteracted by an adaptive compensatory antioxidant defense mechanism in the acute phase of stress. The shift toward the oxidant levels in the absence of an antioxidant stabilizer leads to oxidative stress, impairing brain endothelial function. The aim of this study was to determine the kinetics of oxidative production and changes in activity of the main antioxidant enzymes, superoxide dismutase (SOD) and catalase, after exposure of primary human brain

Abbreviations: BBB, blood–brain barrier; EtOH, ethanol; 4MP, 4-methylpyrazole; Ach, acetaldehyde; ALC, acetyl-L-carnitine; Rosi, rosiglitazone; AP, allopurinol; APC, apocyanin; DDC, diethyldithiocarbamate.

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microvascular endothelial cells to alcohol. We also explored the potential beneficial effects of a mitochondrial protective agent, acetyl-L-carnitine (ALC), and stimulation of nuclear peroxisome proliferator-activated receptor γ (PPARγ) in protecting the BBB via interference with oxidative stress processes. We show that application of ALC in vitro and in vivo mitigates alcohol-induced ROS/RNS production and oxidative damage in the brain [1–4].

A significant body of evidence links PPARγ to the regulation of inflammatory responses, similar to those induced by EtOH in endothelium. PPARγ suppresses gene expression in a ligand-dependent manner by antagonizing the activities of other signal-dependent transcription factors, such as the NF-κB pathway [9–11]. We demonstrated that rosiglitazone (Rosi), an agonist for PPARγ, inhibited immune cell adhesion and migration across the BBB [12] via suppression of Rho GTPases. Furthermore, Rosi was shown to suppress ROS/RNS formation in the brain [13]. In the current study, we aimed to understand antioxidant systems activated by alcohol in brain endothelium and whether ALC or PPARγ activation can protect BBB injury in EtOH exposure.

Although ALC is often considered an indirect antioxidant, neither ALC nor Rosi is a direct scavenger of ROS and RNS. One of the possible mechanisms is that ALC or Rosi may stabilize the function of naturally occurring antioxidants in the brain endothelium, such as SOD and peroxisomal catalase, in alcohol-induced stress. We examined the protective mechanisms of ALC and the anti-inflammatory agent Rosi on the ROS-neutralizing enzymes SOD and catalase during alcohol exposure. We found that both ALC and Rosi increased production of NO, potentially improving brain microvascular tone otherwise affected by EtOH.

Materials and methods

Human brain endothelial cell (hBEC) culture

Primary hBECs were isolated from the temporal cortex of brain tissue that was obtained during surgical removal of epileptogenic foci in adult patients and were supplied by Dr. M. Witte (University of Arizona). Purity evaluation and cell culture conditions for hBECs were performed as described [1]. Cell culture plates and glass coverslips were precoated with type 1 rat-tail collagen (0.09 mg/ml in 0.02 N acetic acid). The excess collagen was aspirated and the plates were dried overnight in a sterile hood. Cells were cultured in DMEM/F-12 medium containing 10 mM Hepes, 13 mM sodium bicarbonate (pH 7), 10% fetal bovine serum, penicillin and streptomycin (100 μg/ml each; Invitrogen), and 50 μg/ml endothelial cell growth supplement (Fisher, Pittsburg, PA, USA) and heparin. Cells were cultured in 96-well plates (20,000 cells/well) for viability and SOD activity assays, in 12-well plates containing glass coverslips (40,000 cells/well) for immunohistochemistry, in 6-well plates (100,000 cells/well) for catalase activity assay, and in T75 (75-cm^2) flasks (1×10^6 cells/flask) for protein extractions. Cell culture media were changed every 3 days until tight monolayers were formed in about 6–7 days.

Before the assay of catalase and SOD activity, dose- and time-dependent effects of ethanol on cell viability were first established using 0–100 mM EtOH for 24- to 72-h exposure time. Ethanol concentrations higher than 50 mM showed toxicity on hBECs after 48 h exposure. We used 20 mM EtOH for the long-term exposure study because it was not directly toxic to endothelial cells for up to 240 h. A concentration of 50 mM EtOH began to show toxicity after 96 h exposure; therefore 50 mM was not ideal to treat hBECs for up to 240 h. The rationale for long-term EtOH exposure is described under Results.

Catalase activity

Once tight monolayers were formed, hBECs were treated with 20 mM EtOH or 50 μM Ach with or without test compounds for 1- to 24-h time points. Cells were detached using a rubber policeman (without inhibitors of proteolytic enzymes), sonicated in 1 ml ice-cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA), and centrifuged at 10,000 *g* for 15 min at 4 °C. Using a Cayman assay kit (Cayman Chemical Co., Ann Arbor, MI, USA), catalase activity was assayed in cell lysate protein (40 μg/replicate) within 2 h of extraction. This assay utilizes the reaction of catalase with methanol in the presence of an optimal H_2O_2 concentration. The end-product, formaldehyde, forms a complex with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as chromogen. The oxidized purple color was measured spectrophotometrically at 540 nm using a SpectraMax M5E microplate reader (Molecular Devices, Sunnyvale, CA, USA). The enzyme activity was extrapolated from a catalase standard curve derived from various concentrations (0–75 μM) of formaldehyde. Results were expressed as nmol/min/ml.

SOD activity

Upon reaching confluency, cell cultures in 96-well plates were exposed to varying concentrations of EtOH at various time points followed by SOD activity determination using the colorimetric SOD assay kit (Cayman Chemical Co.). This SOD kit utilizes the water-soluble tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt, which produces a highly water soluble formazan dye upon reduction with superoxide anions. The rate of reduction with O_2^- is linearly related to xanthine oxidase (XOX) activity, and it is inhibited by SOD. This assay system has the advantage of determining SOD activity over a wide range. Absorbance was read at 450 nm using the SpectraMax M5E microplate reader. SOD activity was expressed as percentage of XOX activity inhibition.

ROS detection

The levels of ROS were detected by dichlorofluorescein diacetate (DCF-DA) assay (Molecular Probes, Eugene, OR, USA) as described previously [3]. Brain endothelial cells cultured in 96-well plates (20,000 cells/well) at the given time points were loaded with 10 μM DCF-DA for 40 min at 37 °C in 200 μl of cell culture medium without phenol red in the presence or absence of inhibitor. After the excess DCF-DA was washed off, the cells were stimulated with test compounds with and without inhibitor for the periods described. Fluorescence intensity was read at various time points with excitation at 488 nm and emission at 525 nm in an ELISA plate reader. Results were expressed as mean relative fluorescence units/mg cellular protein.

NO detection

Following our previously established method, nitric oxide levels were detected by diaminofluorescein-2 diacetate (DAF-2DA) assay (Molecular Probes) [3]. Cleavage of the acetate group from DAF-2DA by intracellular esterases produces a highly fluorescent DAF detected with excitation at 488 nm and emission at 515 nm. Briefly, cells cultured in 96-well plates at the given time points were loaded with 10 μM DAF-2DA for 40 min at 37 °C in 200 μl of phenol red-free cell culture medium with or without inhibitor. After removal of excess DAF-2DA, hBECs were treated with test compounds with or without inhibitor followed by fluorescence readings with excitation at 488 nm and emission at 515 nm. Standard curves were generated with 1, 5, 10, 20, 50, and 100 μM *S*-nitroso-*N*-acetylpenicillamine. Results were expressed as mean relative fluorescence intensity units/mg cellular protein.

Immunofluorescence

Immunohistochemical staining of specific mitochondrial membrane marker proteins assessed the alterations in mitochondrial