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Original article

Alcohol-induced impairment of neuronal nitric oxide synthase (nNOS)-dependent dilation of cerebral arterioles: role of NAD(P)H oxidase

Hong Sun *, Elizabeth Molacek, Hong Zheng, Qin Fang, Kaushik P. Patel, William G. Mayhan

Department of Cellular and Integrative Physiology, 985850 University of Nebraska Medical Center, Omaha, NE 68198-5850

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Abstract

The goal of the present study was to determine the role of NAD(P)H oxidase in alcohol consumption-induced impairment of nNOS-dependent reactivity in cerebral arterioles. Sprague–Dawley rats were fed an alcohol diet for 2–3 months. We measured the effects of acute (1 hour) and chronic (1 month) treatment with a NAD(P)H oxidase inhibitor, apocynin, on responses of parietal pial arterioles to nNOS-dependent agonists (NMDA and kainate) and an nitric oxide synthase (NOS)-independent agonist (nitroglycerin). In addition, we measured the expression of NAD(P)H oxidase subunits and superoxide production in parietal cortex. Topical application of NMDA and kainate produced dose-related dilation of pial arterioles. However, the magnitude of vasodilation to these agonists was significantly less in alcohol-fed rats. Treatment with apocynin (acute and chronic) did not alter vasodilation in nonalcohol-fed rats, but significantly improved vasodilation in alcohol-fed rats. Response of pial arterioles to nitroglycerin was similar in nonalcohol-fed and alcohol-fed rats, and was not affected by apocynin. In addition, we found an up-regulation of gp91phox and p47phox in parietal cortex of alcohol-fed rats. Finally, alcohol consumption produced an increase in superoxide production under basal conditions and in the presence of NADPH. Acute treatment with apocynin suppressed alcohol consumption-induced superoxide generation. Our findings suggest that NAD(P)H oxidase plays an important role in chronic alcohol consumption-induced impairment of nNOS-dependent dilation of cerebral arterioles.

Keywords: Arteries; Oxygen radicals; Microcirculation; NAD(P)H oxidase; Nitric oxide

1. Introduction

Chronic consumption of alcohol and binge drinking can contribute to the pathogenesis of cerebrovascular disorders, including hemorrhagic and ischemic stroke [1–5]. In previous studies, we found that acute [6] and chronic [7–9] exposure to alcohol impairs nNOS- and endothelial nitric oxide synthase (eNOS)-dependent dilation of cerebral arterioles. Mechanisms that contribute to impaired nNOS- and eNOS-dependent dilation of cerebral arterioles during alcohol consumption are not entirely clear, but appear to involve the formation of reactive oxygen species. In our previous studies, we found that topical application with glutathione and SOD partially restored impaired nNOS- and eNOS-dependent vasodilation of pial arterioles, respectively [10,11]. However, the precise cellular pathways that account for increased production of oxygen radicals during alcohol consumption are not known. NAD(P)H oxidase is major source of oxygen radicals, and a recent study suggests that NAD(P)H oxidase may play a key role in alcohol-induced liver disease [12]. No studies that we are aware of have examined the role of NAD(P)H oxidase in impaired responses of cerebral arterioles during chronic consumption of alcohol. Thus, the goal of the present study was to determine whether NAD(P)H oxidase is involved in alcohol consumption-induced impairment of nNOS-dependent cerebral vasodilation.

* Corresponding author. Tel.: +402 5 59 5040; fax: +402 5 59 4438.
E-mail address: hsu1@unmc.edu (H. Sun).

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2. Methods

2.1. Experimental diets

We used male Sprague–Dawley rats. At about 2 months of age (body weight 200–220 g), the rats were divided into four groups: nonalcohol-fed ($N = 8$), nonalcohol-fed apocynin treated ($N = 8$), alcohol-fed ($N = 8$), and alcohol-fed apocynin treated ($N = 8$). We fed rats liquid diets (Dyets, Bethlehem, PA) for 2–3 months. The nonalcohol-fed rats were given a diet that contained 1.0 kcal/ml, of which 35% are derived from fat, 47% are derived from carbohydrates, and 18% are derived from protein. Rats in the alcohol-fed groups were given a diet that contained 1.0 kcal/ml, of which 35% are derived from fat, 11% are derived from carbohydrates, 18% are derived from protein, and 36% are derived from ethanol. The ethanol was gradually introduced into the diet, and the total daily volume of diet fed to the nonalcohol-fed rats was based on the consumption of diet by the alcohol-fed rats. We have shown previously that the alcohol-containing diet produces a plasma alcohol concentration of about 20 mmol/l in rats [7,10]. In chronic studies, apocynin (7.5 mg/kg per day in the diet) was started 1 month before the day of the experiment.

2.2. Preparation of animals

On the day of the experiment, the rats were anesthetized (thiobutabarbital sodium (Inactin), 100 mg/kg body weight, ip), and a tracheotomy was performed. The rats were ventilated mechanically with room air and supplemental oxygen. A catheter was placed into a femoral vein for injection of supplemental anesthesia, and a femoral artery was cannulated for measurement of arterial blood pressure and to obtain a sample for the measurement of arterial blood gas.

To visualize the microcirculation of the cerebrum, a craniectomy was prepared over the left parietal cortex. The cranial window was suffused with artificial cerebrospinal fluid (2 ml/min) that was bubbled with 95% nitrogen and 5% carbon dioxide. Temperature of the suffusate was maintained at 37 ± 1 °C. The cranial window was connected via a three-way valve to a pump, which allowed for infusion of agonists and antagonists into the suffusate. This method maintained a constant temperature, pH, pCO_2 and pO_2 of the suffusate during infusion of drugs. Diameter of pial arterioles was measured using a video image-shearing device (model 908, Instrumentation for Physiology and Medicine, Inc.).

2.3. Experimental protocol

The cranial window was superfused with artificial cerebral spinal fluid for 1 hour before testing responses of arterioles. Responses of pial arterioles were examined during suffusion of agonists that presumably produce vasodilation via activation of nNOS (NMDA (100 and 300 μmol/l) and kainate (100 and 300 μmol/l)). We also examined responses of pial arterioles to nitroglycerin (0.01 and 0.1 μmol/l), which presumably produces vasodilation independent of NOS. Agonists were mixed in artificial cerebral spinal fluid, and then superfused over the cranial window in a random manner. Diameter of pial arterioles was measured immediately before application of agonists and every minute for 5 min during application of agonists. Steady-state responses to the agonists were reached within 2–3 min after starting application and the diameter of pial arterioles returned to baseline within 5 min after application of agonists was stopped.

In acute studies, after initially examining responses of pial arterioles to the agonists, we then examined the effect of suffusion with apocynin (1 mmol/l) on responses of pial arterioles in nonalcohol-fed and alcohol-fed rats. One hour after the suffusion of apocynin was started, and continuing for the duration of the experiment, we again examined responses of pial arterioles to the agonists.

After testing responses of pial arterioles, rats from nonalcohol-fed and alcohol-fed groups were sacrificed by phlebotomy. The brains were immediately extracted, cleaned with phosphate-buffer solution (PBS). Parietal cortex tissue was collected, and stored at –80 °C until Western blot and Real-Time PCR analysis.

2.4. Western blot

Samples were homogenized in 20% (weight/volume) ice-cold buffer containing 10 mmol/l Tris–HCl, pH 7.4; 1% SDS; 1 mmol/l sodium vanadate; 10 μg/ml aprotinin; 10 μg/ml leupaptin; and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF). Next, samples were centrifuged at 12,000 × g for 20 min at 4 °C and protein concentration in the supernatant was determined by the Bradford method (Bio-Rad, Richmond, CA) with BSA as the standard. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 7.5% or 12.5% gel on which 15 μg of total protein/well was loaded. After SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane. Immol/Lunoblotting analysis was performed using goat anti-NAD(P)H oxidase subunits (gp91phox, p47phox, p67phox, and Nox1) and rabbit anti-NAD(P)H oxidase subunit (p22phox) (Santa Cruz Biotechnology) as the primary antibody and peroxidase conjugated rabbit anti-goat or goat anti-rabbit IgG as the second antibody. The bound antibody was detected using the ECL kit and quantified by densitometry. The amount of target proteins in alcohol-fed rats was expressed as percent relative to that in nonalcohol-fed rats.

2.5. Real-time PCR

Total RNA was extracted according to the TRI Reagent manufacturer's instructions, and subjected to reverse transcription for 40 min at 37 °C in the presence of 1.5 μmol/l of random hexamers and 100 units of MMLV-RTase. The cDNA was amplified in a real time PCR apparatus (iCycler, Bio-Rad Laboratories), in the presence of 1X QuantiTect Sybr