Role of NAD(P)H Oxidase in Alcohol-Induced Impairment of Endothelial Nitric Oxide Synthase–Dependent Dilation of Cerebral Arterioles

Hong Sun, PhD; Hong Zheng, MD; Elizabeth Molacek; Qin Fang, MD; Kaushik P. Patel, PhD; William G. Mayhan, PhD

Background and Purpose—Our goal was to determine whether NAD(P)H oxidase is involved in impaired endothelial nitric oxide synthase (eNOS)–dependent reactivity of cerebral arterioles during chronic alcohol consumption.

Methods—Sprague-Dawley rats were fed with an alcohol diet for 2 to 3 months. We determined the effects of acute and chronic treatment with an NAD(P)H oxidase inhibitor, apocynin, on responses of pial arterioles to eNOS-dependent agonists (acetylcholine and ADP) and an eNOS-independent agonist (nitroglycerin). Expression of NAD(P)H oxidase in pial arterioles was measured with the use of real-time polymerase chain reaction and Western blot analysis, and superoxide production was measured with the use of lucigenin-enhanced chemiluminescence.

Results—Vasodilation in response to acetylcholine and ADP, but not nitroglycerin, was significantly less in alcohol-fed rats. Treatment with apocynin did not alter vasodilation in non–alcohol-fed rats but significantly improved impaired vasodilation in alcohol-fed rats. In addition, an upregulation of p47phox in pial arterioles was found in alcohol-fed rats. Furthermore, alcohol consumption increased superoxide production under basal conditions and in the presence of ADP and NAD(P)H.

Conclusions—Our findings suggest that NAD(P)H oxidase plays a role in chronic alcohol consumption–induced impairment of eNOS-dependent dilation of cerebral arterioles. (Stroke. 2006;37:495-500.)

Key Words: alcohol ▪ NADPH oxidase ▪ nitric-oxide synthase ▪ stroke

Chronic consumption of alcohol and binge drinking are contributing factors to hemorrhagic and ischemic stroke.1-3 Alcohol consumption may predispose to cerebrovascular dysfunction by altering responses of cerebral vessels to activation of important vasodilator pathways. In previous studies we found that dilation of cerebral arterioles to agonists that stimulate endothelial and/or neuronal synthesis/release of nitric oxide (NO) is profoundly impaired during alcohol consumption.4,5 In addition, vasodilation to activation of potassium channels and adénylate cyclase (via β-adrenergic receptors) is impaired in alcohol-fed rats.5 Thus, several cellular pathways that account for dilation of pial arterioles are altered during chronic consumption of alcohol.

Mechanisms that account for impaired endothelial NO synthase (eNOS)–dependent dilation of cerebral arterioles during alcohol consumption are not entirely clear. In a previous study we found that impairment of eNOS-dependent vasodilation could be prevented by superoxide dismutase.7 We also found an increased plasma level of lipid peroxidation in alcohol-fed rats.7 These findings suggest that reactive oxygen species, presumably superoxide, plays a role in impaired eNOS-dependent dilation of cerebral arterioles during alcohol consumption. In addition to inactivation of NO, reactive oxygen species oxidize tetrahydrobiopterin (BH₄),8 thereby increasing superoxide and reducing NO bioavailability. We found that treatment with BH₄ restored eNOS-dependent vasodilation in alcohol-fed rats, suggesting an alteration/deficiency/uncoupling in the utilization of BH₄ favoring an increase in the production of superoxide over NO.9 However, the precise cellular pathway responsible for increased oxidative stress during alcohol consumption is not known.

NAD(P)H oxidase is a major source of oxygen radicals and may play a role in alcohol-induced liver disease.10 However, no studies have examined the role of NAD(P)H oxidase in impaired responses of cerebral arterioles during chronic consumption of alcohol. Thus, our goal was to determine whether NAD(P)H oxidase is involved in alcohol consumption–induced impairment of eNOS-dependent cerebral vasodilation.

Methods

Experimental Diets

We used male Sprague-Dawley rats (Sasco, Wilmington, Mass). At ~2 months of age (body weight, 200 to 220 g), the rats were divided into 4 groups: non–alcohol-fed (n = 14), non–alcohol-fed apocynin (n = 14), alcohol-fed (n = 8), and alcohol-fed apocynin (n = 8) rats. We fed rats liquid diets (Dyets; Bethlehem, Pa) for 2 to 3 months as decided previously.7,9 In some studies, apocynin (7.5 mg/kg per day in the diet) was started 1 month before the day of the experiment.
Preparation of Animals

On the day of the experiment, the rats were anesthetized (thiobutabarbital sodium [Inactin], 100 mg/kg body wt 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 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 3-way valve to a pump, which allowed for infusion of agonists and antagonists. Diameter of pial arterioles was measured with the use of a video image-shearing device.

Experimental Protocol

Responses of pial arterioles were examined during suffusion of agonists that presumably produce vasodilation via activation of eNOS (acetylcholine [0.1 and 1 μmol/L] and ADP [1 and 10 μmol/L]). We also examined responses to nitroglycerin (0.01 and 0.1 μmol/L), which produces vasodilation independent of eNOS. Agonists were mixed in artificial cerebrospinal fluid and then superfused over pial microcirculation in a random manner. Diameter of pial arterioles was measured immediately before application of agonists and every minute for 5 minutes during application of agonists. Steady state responses were reached within 2 to 3 minutes, and the diameter returned to baseline within 5 minutes after application of agonists was stopped.

In acute studies, after initially examining responses to the agonists, we then examined the effect of treatment with apocynin (1 mMol/L) in non–alcohol-fed and alcohol-fed rats. One hour after the suffusion of apocynin was started and for the duration of the experiment, we examined responses of pial arterioles to the agonists. After functional responses were examined, the rats were euthanized by phlebitomy. The brains were extracted and cleaned with PBS. Pial arterioles from both hemispheres were isolated and stored at −80°C until Western blot and Real-time polymerase chain reaction (PCR) analysis.

Western Blot

Samples were homogenized in 20% (wt/vol) 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 leupeptin; and 1 mMol/L, phenylmethylsulfonyl fluoride. Next, samples were centrifuged at 12,000 g for 20 minutes at 4°C, and protein concentration in supernatant was determined by the Bradford method (Bio-Rad) with BSA as the standard. SDS-PAGE was performed on a 7.5% or 12.5% gel on which 15 μg of total protein per well was loaded. After SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride membrane. Immunoblotting analysis was performed with the use of goat anti-NAD(P)H oxidase subunits (gp91phox, mox-1, p47phox, p67phox, and p22phox) (Santa Cruz Biotechnology) as primary antibody and peroxidase-conjugated secondary antibody. The membranes were exposed for imaging. Densitometry was used to quantify, target gene expression was normalized to the expressed housekeeping gene RPL19. The sequence of the oligonucleotide primers was as follows: p47phox, 5′-ACCTACAGCTGGTAGATTCTCACA-3′ and 3′-TCATCGGGGCCGACTTT-5′ (GenBank AF260779); p67phoxphox, 5′-GCTTCGGAACCATGGTGCTAAGA-3′ and 3′-AGATGACGGCAGAGTTTTCTACTG-5′ (GenBank AB2002664); p22phox, 5′-ACCTACAGCTGGTAGATTCTCACA-3′ and 3′-GTTGAGACAGGCCGGGA-5′ (GenBank AJ295951); RPL19, 5′-CCCAAAATGAAAAAACGAAA-3′ and 3′-ATGGACATCGACAGGCTCT-5′ (GenBank NM031103).

Superoxide Measurement

Superoxide production was measured with the use of lucigenin-enhanced chemiluminescence. After the rat was exsanguinated, the brain was removed and immersed in cold modified Krebs-HEPES buffer containing the following (in mMol/L): 118 NaCl, 4.7 KCl, 1.1 CaCl2, 1.2 MgCl2, 1.2 KH2PO4, 25 NaHCO3, 10 HEPES, 10 glucose (pH 7.4). The middle cerebral artery (MCA) was isolated, and adventitial tissue was removed. Ring segments of MCA were included in polypropylene tubes containing 5 μmol/L lucigenin, then read in a Femtomaster FB12 (Zytox) luminometer, which reports relative light units emitted integrated over 30-second intervals for 5 minutes. Data were corrected for background and normalized to total protein of MCA segments.

Statistical Analysis

Results were compared with a 2-way repeated-measures ANOVA with Tukey post hoc test. Student t tests were used to compare responses to agonists before and after application of apocynin. Values are mean±SEM. A probability value of ≤0.05 was considered significant.

Results

Control Conditions

Body weight and mean arterial pressure were similar in all groups (non–alcohol-fed: 402±4 g, 113±7 mm Hg; alcohol-fed: 391±9 g, 104±6 mm Hg; non–alcohol-fed apocynin: 409±9 g, 112±3 mm Hg; alcohol-fed apocynin: 392±10 g, 106±3 mm Hg).

Responses to Agonists

Baseline diameter of pial arterioles was 38±2 μm in non–alcohol-fed and 38±1 μm in alcohol-fed rats (P>0.05). Acetylcholine (Figure 1) and ADP (Figure 2) produced dilation of pial arterioles in all groups. However, the magnitude of vasodilation was significantly less in alcohol-fed than in non–alcohol-fed rats. In contrast, alcohol consumption did not alter responses to nitroglycerin (Figure 3).

Responses After Topical Application of Apocynin

Topical application of apocynin did not alter baseline diameter of pial arterioles in non–alcohol-fed and alcohol-fed groups. In addition, apocynin did not alter dilation of pial arterioles to acetylcholine, ADP, and nitroglycerin in non–alcohol-fed rats (Figures 1, 2, and 3). In contrast, apocynin significantly improved dilation of pial arterioles to acetylcholine and ADP in alcohol-fed rats (Figures 1, 2, and 3).

Responses After Chronic Treatment With Apocynin

Baseline diameter of pial arterioles was 38±1 μm in non–alcohol-fed apocynin and 38±2 μm in alcohol-fed apocynin rats (P>0.05). Application of acetylcholine, ADP, and nitroglycerin dilated pial arterioles in non–alcohol-fed apocyninn-treated and alcohol-fed apocynin-treated rats. Compared with that observed in alcohol-fed rats, impaired vasodilation to acetylcholine and ADP was alleviated in alcohol-fed apocynin-treated rats (Figures 1 and 2). Treatment of non–
alcohol-fed rats with apocynin did not alter vasodilation to acetylcholine, ADP, or nitroglycerin (Figures 1, 2, and 3).

Expression of NAD(P)H Oxidase Subunits
Chronic consumption of alcohol did not alter expression of mox-1, p22phox, and p67phox but significantly upregulated p47phox in parietal pial arterioles (Figures 4 and 5). We did not detect gp91phox in pial arterioles.

Superoxide Production
Basal superoxide was significantly higher in the MCA of alcohol-fed than in non–alcohol-fed rats (Figure 6). ADP (100 μmol/L) enhanced superoxide production in the MCA of alcohol-fed but not non–alcohol-fed rats (Figure 6). In addition, NAD(P)H (10 μmol/L)-induced superoxide production was significantly higher in alcohol-fed rats. Preincubation of the MCA with apocynin (1 mmol/L for 1 hour) did not affect superoxide production in non–alcohol-fed rats but markedly reduced superoxide production under basal conditions and in the presence of ADP and NAD(P)H in alcohol-fed rats.

Discussion
There are 3 new findings from this study. First, acute and chronic treatment with apocynin improves alcohol consumption–induced impairment of eNOS-dependent dilation of pial arterioles.
arterioles. Second, alcohol consumption produces an upregulation in p47phox in pial arterioles.

Third, alcohol consumption increases superoxide production under basal conditions and in the presence of ADP and NAD(P)H, and apocynin can suppress alcohol consumption—induced superoxide generation. We suggest that impaired eNOS-dependent dilation of pial arterioles during alcohol consumption is related to an increased expression/activity of NAD(P)H oxidase.

Several studies found that chronic exposure to alcohol results in an impairment of eNOS-dependent reactivity in large peripheral blood vessels of animals and humans. We found that chronic alcohol consumption also impairs eNOS-dependent dilation of pial arterioles. To determine the effect of alcohol consumption on eNOS-dependent reactivity in pial arterioles, we examined responses to acetylcholine and

![Figure 3](image3.png)

**Figure 3.** Effects of chronic (7.5 mg/kg per day for 1 month) and acute (1 mmol/L for 1 hour) treatment with apocynin on response of pial arterioles to nitroglycerin in 2- to 3-month non-alcohol-fed (control) and alcohol-fed rats (alcohol). Values are mean±SE.

![Figure 4](image4.png)

**Figure 4.** Assessment of the effect of chronic alcohol consumption on NAD(P)H oxidase subunits in rat pial arterioles. A, Representative Western immunoblots of NAD(P)H oxidase subunit proteins (lanes 1 to 4, control; lanes 5 to 8, alcohol). B, Quantified data from the Western blots. Values are mean±SE. *P<0.05 vs control.

![Figure 5](image5.png)

**Figure 5.** Calculated NAD(P)H oxidase subunits/RPL19 mRNA ratios in pial arterioles of non-alcohol-fed (control) and alcohol-fed (alcohol) rats. Quantification of mRNA expression was performed by real-time PCR with the Bio-Rad Multicolor Real-Time PCR Detection System. *P<0.05 vs control.
ADP. Previous studies have shown that dilation of rat pial arterioles in response to acetylcholine and ADP, but not nitroglycerin, could be attenuated by application of enzymatic inhibitors of NOS.\(^{14,15}\) In addition, Xu et al\(^{16}\) reported that NOS inhibitor \(N^\circ\)-nitro-l-arginine methyl ester but not neuronal NOS–selective inhibitor ARR-17477 significantly reduced acetylcholine- and ADP-induced dilation of pial arterioles in rats. These findings suggest that dilation of rat pial arterioles in response to acetylcholine and ADP is related to the endothelial synthesis/release of NO.

Previous studies,\(^{17,18}\) including ours,\(^{7}\) found that alcohol induces oxidative stress. In addition, impaired eNOS-dependent cerebral vasodilation during alcohol consumption can be restored by scavenging oxygen radicals.\(^{7,19}\) Thus, we suggest that alcohol consumption impairs eNOS-dependent responses of cerebral arterioles via an increase in oxidative stress, presumably an increase in the production of superoxide. However, cellular networks responsible for the formation of superoxide during alcohol consumption are not entirely clear. NAD(P)H oxidase has been found to contribute to vascular dysfunction during several disease states.\(^{20,21}\) In addition, Kono et al\(^{10}\) reported that NAD(P)H oxidase–derived oxygen radicals are key oxidants in alcohol-induced liver disease. In the present study we found that chronic consumption of alcohol increased superoxide production. Furthermore, treatment with apocynin suppressed alcohol consumption–induced superoxide production, suggesting that increased oxidative stress during alcohol consumption may be related to activation of NAD(P)H oxidase. Recent studies have used apocynin to examine the effects of oxidative stress, via NAD(P)H oxidase, on vascular dysfunction during a variety of disease states.\(^{22,23}\) In addition to measuring superoxide production, we also conducted functional studies to examine the role of NAD(P)H oxidase in impaired responses of pial arterioles to eNOS-dependent agonists during alcohol consumption and biochemical studies to determine the influence of alcohol consumption on subunits of NAD(P)H oxidase. We found that acute and chronic treatment with apocynin alleviated impaired eNOS-dependent reactivity of pial arterioles in alcohol-fed rats. We also found that alcohol consumption upregulated the expression of p47phox in pial arterioles. These findings suggest that NAD(P)H oxidase plays an important role in alcohol consumption–induced oxidative stress and impairment of eNOS-dependent reactivity of cerebral arterioles.

NAD(P)H oxidase is an important source of superoxide in cells that constitute the vascular wall, including endothelial and vascular smooth muscle cells.\(^{24,25}\) NAD(P)H oxidase consists of 4 major subunits: gp91phox, p22phox, p47phox, and p67phox. In some nonphagocytic cells there are gp91phox homologues that serve a similar function. A homologue of gp91phox, mox-1, was identified in rat aortic smooth muscle cells.\(^{26}\) In the present study mox-1, but not gp91phox, was detected in pial arterioles and was not altered by alcohol consumption. However, we found an upregulation of p47phox in pial arterioles of alcohol-fed rats. The function of p47phox is to facilitate steady association of gp91phox and p67phox.\(^{27}\) Activated enzyme complex then uses NAD(P)H as the electron donor for reduction of molecular oxygen to superoxide. It has been demonstrated that p47phox is critical for vascular NAD(P)H oxidase activation.\(^{28}\) Previous studies found that p47phox is upregulated in diseased vessels.\(^{28,29}\) The precise cellular pathway(s) underlying increased p47phox expression remains uncertain. Angiotensin II and thrombin have been reported to increase vascular p47phox expression.\(^{28,29}\) Alcohol consumption robustly activates the renin-angiotensin system and increases plasma concentration of angiotensin II.\(^{30}\) Thus, the mechanism underlying increased expression of p47phox during chronic alcohol consumption requires further investigation.

In the present study we found an increased superoxide production under basal conditions and in the presence of ADP and NAD(P)H, which could be suppressed by acute treatment with apocynin. These findings suggest that increased superoxide generation during alcohol consumption is related to NAD(P)H oxidase. Although an increased superoxide production was found, it is still not clear which cell line(s) is responsible for increased superoxide production during alcohol consumption. In addition, we used the MCA to examine superoxide production.
Although it is not known whether there are segmental differences in the production of superoxide, we suggest that the MCA may be representative of cerebral arterioles. Furthermore, although numerous studies have used a concentration of lucigenin similar to the present study, others\(^8\) have suggested that lucigenin may influence superoxide production. In the present study ADP did not affect superoxide in non–alcohol-fed rats but significantly enhanced superoxide production in alcohol-fed rats. It seems that eNOS might be a potential source of superoxide during alcohol consumption. A recent study demonstrated that NAD(P)H oxidase produces superoxide and thus leads to oxidation of BH\(_4\), which decreases NO production and increases superoxide production from eNOS in aorta of hypertensive rats.\(^8\)

In a previous study we found that topical application of BH\(_4\) restored impaired eNOS-dependent dilation of pial arterioles during alcohol consumption. Thus, it is possible that an increased superoxide production via NAD(P)H oxidase during chronic alcohol consumption induces an oxidation of BH\(_4\), and thus further impairs eNOS-dependent cerebral vasodilation.

### Summary

In summary, we examined the role of NAD(P)H oxidase in chronic alcohol consumption–induced impairment of eNOS-dependent reactivity of pial arterioles. We found that consumption of alcohol impairs reactivity of pial arterioles to acetylcholine and ADP but not to nitroglycerin. In addition, we found that superoxide production and the expression of p47phox were increased in alcohol-fed rats. Furthermore, acute and chronic treatment with an inhibitor of NAD(P)H oxidase alleviated alcohol-induced impairment of eNOS-dependent reactivity. We suggest that superoxide released via activation of NAD(P)H oxidase contributes to impaired eNOS-dependent dilation of pial arterioles during alcohol consumption. We speculate that our findings may have important implications for the pathogenesis of cerebrovascular abnormalities, including stroke, in chronic alcoholics.

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