Tetrahydrobiopterin, a cofactor for NOS, improves endothelial dysfunction during chronic alcohol consumption

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Sun, Hong, Kaushik P. Patel, and William G. Mayhan. Tetrahydrobiopterin, a cofactor for NOS, improves endothelial dysfunction during chronic alcohol consumption. Am J Physiol Heart Circ Physiol 281: H1863–H1869, 2001.—We sought to investigate mechanisms that may account for impaired nitric oxide synthase (NOS)-dependent dilatation of cerebral arterioles during alcohol consumption. Our goals were to examine 1) the effect of exogenous application of a cofactor for NOS, i.e., tetrahydrobiopterin (BH4) on the reactivity of pial arterioles during alcohol consumption; and 2) endothelial NOS (eNOS) protein in nonalcohol-fed and alcohol-fed rats. Sprague-Dawley rats were fed liquid diets with or without alcohol for 2–3 mo. We measured in vivo diameter of pial arterioles in response to NOS-dependent agonists (ACh and ADP) and a NOS-independent agonist (nitroglycerin) before and during application of BH4. Blood vessels were then harvested for Western blot analysis of eNOS protein. In nonalcohol-fed rats, ACh and ADP produced vasodilatation, which was impaired in alcohol-fed rats. Vasodilatation to nitroglycerin was similar in both groups of rats. Application of BH4 did not alter vasodilatation in nonalcohol-fed rats but improved impaired vasodilatation in alcohol-fed rats. Also, eNOS protein in cerebral cortex microvessels, the basilar artery, and aorta was not different between nonalcohol-fed and alcohol-fed rats. Thus impaired NOS-dependent vasodilatation during alcohol consumption does not appear to be related to an alteration in eNOS protein but may be related to a deficiency and/or alteration in the utilization of BH4

rats; reactivity; brain; cerebral arterioles; stroke; acetylcholine; adenosine 5'-diphosphate; nitroglycerin; endothelial nitric oxide synthase protein

PROLONGED, HEAVY CONSUMPTION of alcohol and binge drinking can contribute to the pathogenesis of cerebrovascular disorders, including hemorrhagic and ischemic stroke (6, 9, 10, 23, 34, 46). In previous studies, we have shown that acute (19) and chronic (18, 20, 32, 33) exposure to alcohol impairs nitric oxide synthase (NOS)-dependent reactivity of large and small cerebral blood vessels. From these previous studies, we have suggested that the pathogenesis of cerebrovascular abnormalities during alcohol consumption may be related to an impairment in dilator responses of cerebral blood vessels.

In addition to examining reactivity of large and small cerebral blood vessels, we (32, 33) have also investigated mechanisms that may contribute to impaired responses of cerebral vessels during chronic alcohol consumption. We found that topical application of superoxide dismutase could ameliorate impaired NOS-dependent dilatation of pial arterioles (33) and the basilar artery (32). Thus we suggested that a potential mechanism of impaired NOS-dependent dilatation of cerebral blood vessels during alcohol consumption may be related to an increased release of oxygen-derived free radicals, presumably superoxide anion. However, the potential pathway that accounted for the increase in superoxide anion was not examined. Previous studies have suggested that under various conditions activation of endothelial NOS (eNOS) can produce both nitric oxide and superoxide anion (26, 31, 39, 40). It appears that the metabolism of tetrahydrobiopterin (BH4) may play a key role in the control of production of nitric oxide and superoxide anion by eNOS (1, 27, 31, 39, 40). An insufficiency or impaired metabolism of BH4 may lead to uncoupling of the L-arginine-nitric oxide pathway, resulting in increased formation of superoxide anion and reduced nitric oxide production (4, 8, 40, 43). Yoshimoto et al. (44) found that alcohol reduces brain BH4 levels in mice, and thus it is conceivable that a reduction in BH4 could contribute to impaired reactivity of cerebral blood vessels during alcohol consumption. Thus the first goal of this study was to examine the effects of exogenous application of BH4 on NOS-dependent reactivity of pial arterioles.

In addition to an alteration in an important cofactor for the synthesis of nitric oxide, impaired reactivity of cerebral blood vessels during alcohol consumption may be related to an alteration in the NOS system. We have shown that dilatation of cerebral blood vessels to agonists that presumably activate eNOS is impaired during chronic alcohol consumption (18, 20, 32, 33). In addition, previous studies have reported that acute exposure to alcohol increases eNOS protein, activity,
and expression in cultured bovine aortic endothelial cells, ovarian tissue, and human placental villous tissue (12, 16, 30, 41). Because eNOS is a potential source of oxygen radicals, in the presence of suboptimal levels of BH4, increased eNOS protein expression may induce an increased formation and/or release of superoxide anion that may contribute to impaired NOS-dependent dilatation of cerebral blood vessels. However, we are not aware of any studies that have examined eNOS protein in cerebral blood vessels during chronic alcohol consumption. Thus the second goal of the present study was to examine the effect of chronic alcohol consumption on eNOS protein in cerebral blood vessels.

**METHODS**

**Experimental diets.** We used male Sprague-Dawley rats in these studies. At 2 mo of age (body weight = 200–220 g), the rats were randomly divided into two groups: a nonalcohol-fed group (n = 8) and an alcohol-fed group (n = 11). We fed the rats liquid diets (Dyets; Bethlehem, PA) for 2–3 mo. These diets have been used extensively to study the chronic effects of alcohol in rats. The nonalcohol-fed rats were given a liquid diet that did not contain ethanol. This diet contains 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 liquid diet (Dyets) that contained ethanol. This diet contains 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 over an 8-day period, as described previously (18). We measured the daily volume of diet consumed by the rats. The total daily volume of diet fed to the nonalcohol-fed rats was based on the daily consumption of diet by the alcohol-fed rats. Thus the daily consumption of diet was similar in the nonalcohol-fed and alcohol-fed rats.

**Preparation of animals.** Rats were prepared for studies 2–3 mo after starting the nonalcohol or alcohol diets. On the day of the experiment, the rats were anesthetized [thiobutenabarital sodium (Inactin), 100 mg/kg body wt ip], and a tracheotomy was performed. The animals 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 blood sample for the measurement of plasma alcohol.

To visualize the microcirculation of the cerebral, a craniectomy was prepared over the left parietal cortex. The cranial window was suffused with artificial cerebral spinal fluid (2 ml/min) that was bubbled continuously with 95% N2-5% CO2. Temperature of the suffusate was maintained at 37 ± 1°C. The cranial window was connected via a three-way valve to an infusion pump, which allowed for infusion of agonists and BH4 into the suffusate. This method, which we have used previously (18, 32, 33), maintained a constant temperature, pH, PCO2, and PO2 of the suffusate during infusion of drugs. Arterial blood gases were monitored and maintained within normal limits throughout the experiment. Diameter of pial arterioles was measured using a video image shearing device (model 908, Instrumentation for Physiology and Medicine, San Diego, CA).

**Experimental protocol.** The cerebral microcirculation was suffused with artificial cerebral spinal fluid for 1 h before testing responses of arterioles to the agonists. Responses of pial arterioles were examined during superfusion with NO-dependent agonists: acetylcholine (1 and 10 μM) and ADP (10 and 100 μM). We also examined the responses of pial arterioles to nitroglycerin (0.1 and 1.0 μM), which produces cerebral vasodilatation independent of NOS. To examine the effect of exogenous BH4 on responses of pial arterioles during chronic alcohol consumption, we initially examine responses of arterioles to the agonists, and then we suffused the cranial window preparation with cerebral spinal fluid containing BH4 (0.1 μM). One hour after the suffusion of BH4 was started and continued during the experiment, we again examined responses of pial arterioles to the agonists.

Drugs were mixed in artificial cerebral spinal fluid and then superfused over the cerebral microcirculation. Application of agonists was randomized, and in each rat we studied responses of the largest pial arteriole exposed by the craniectomy to application of agonists. The diameter of pial arterioles was measured immediately before application of agonists and every minute during application of agonists. Steady-state response to agonists were reached within 2–3 min after starting application of the agonist, and the diameter of pial arterioles returned to baseline within 3–5 min after application of agonist was stopped.

**Microvessel isolation.** After the experimental protocol was completed, tissue (cerebrum, basilar artery, and aorta) was harvested, rinsed with phosphate-buffer solution (PBS), frozen on dry ice, and stored at −80°C until assessment of eNOS protein using Western blot analysis. Cerebral microvessels were isolated from the rat cerebral cortex using a procedure described previously (21), with slight modifications. The cerebral cortex from one rat was homogenized gently with a Dounce tissue grinder in an ice-cold PBS (0.01 mol/l, pH 7.4). The homogenized brains were then centrifuged at 2,000 g for 10 min at 4°C. The supernatant was discarded, and the pellet was washed by resuspension in PBS and recentrifuged at 2,000 g for 10 min. The supernatant was discarded, and the pellet was washed by resuspension in PBS and recentrifuged at 4,000 g for 20 min. The pellet was then collected, resuspended in the dextran solution, and centrifuged at 4,000 g for 20 min. The final pellet was poured over a nylon mesh screen (50 μm) and washed with a stream of cold PBS. The microvessel fraction was collected from the top of the screen and stored at −80°C.

**Western blot analysis.** Frozen cerebral microvessel samples, basilar arteries, and aortas were homogenized separately in 20% (wt/vol) ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 1% SDS, 1 mM sodium vanadate, 10 μg/ml aprotinine, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 12,000 g for 20 min at 4°C, and the protein concentration in supernatant was determined by the Bradford method (Bio-Rad; Richmond, CA) with bovine serum albumin as the standard. SDS-PAGE was performed on a 7.5% gel on which 1.5 μg of total protein/well was loaded. After SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride membrane. Immunoblot analysis was performed using a mouse monocolonal anti-eNOS as the primary antibody (1:1,000) and a horse radish peroxidase-conjugated goat anti-mouse IgG (1:2,000) as the second antibody. The bound antibody was detected using an ECL kit and quantified by scanning densitometry.

The intensities of the eNOS bands were normalized with respect to the intensities of molecular weight standard bands detected on the same Western blots.

**Statistical analysis.** An unpaired t-test was used to compare values between nonalcohol-fed and alcohol-fed rats in response to the agonists. A paired t-test was used to compare values obtained before and after application of BH4 to the
cerebral microcirculation. A P value of 0.05 or less was considered to be significant. Values are means ± SE.

RESULTS

Control conditions. Body weight in nonalcohol-fed rats was 391 ± 5 g and in alcohol-fed rats was 385 ± 6 g (P > 0.05). Blood alcohol concentration was greater in alcohol-fed rats (20 ± 2 mM) than in nonalcohol-fed rats (0 ± 0 mM) (P < 0.05). Mean arterial pressure was similar in nonalcohol-fed rats and alcohol-fed rats before and during suffusion with BH4. In nonalcohol-fed rats, mean arterial pressure was 85 ± 7 mmHg before suffusion with BH4 and 80 ± 5 mmHg after suffusion with BH4 (P > 0.05). In alcohol-fed rats, mean arterial pressure was 85 ± 6 mmHg before suffusion with BH4 and 80 ± 5 mmHg after suffusion with BH4 (P > 0.05).

Responses to agonists. Baseline diameter of pial arterioles was 41 ± 2 μm in nonalcohol-fed rats and 40 ± 2 μm in alcohol-fed rats (P > 0.05). In the nonalcohol-fed rats, acetylcholine (Fig. 1) and ADP (Fig. 2) produced dose-related dilatation of pial arterioles. In contrast, in alcohol-fed rats, acetylcholine (Fig. 1) produced constriction of pial arterioles, and the magnitude of vasodilatation in response to ADP (Fig. 2) was significantly less than that observed in nonalcohol-fed rats. These findings could not be explained by a nonspecific impairment in vasodilatation in alcohol-fed rats, because nitroglycerin produced similar dose-related dilatation of pial arterioles in nonalcohol-fed and alcohol-fed rats (Fig. 3).

Topical application of BH4 (0.1 μM) did not alter baseline diameter of pial arterioles in nonalcohol-fed rats (40 ± 2 μm before BH4 vs. 44 ± 3 μm after BH4) or alcohol-fed rats (40 ± 2 μm before BH4 vs. 42 ± 2 μm after BH4). In nonalcohol-fed rats, topical application of BH4 did not alter dilatation of pial arterioles in response to acetylcholine (Fig. 1), ADP (Fig. 2), or nitroglycerin (Fig. 3). In contrast, topical application of BH4 significantly improved dilatation of pial arterioles in response to acetylcholine (Fig. 1) and ADP (Fig. 2) in alcohol-fed rats. Topical application of BH4 did not alter dilatation of pial arterioles in response to nitroglycerin (Fig. 3) in alcohol-fed rats.

Expression of eNOS protein. Figure 4 shows Western blot analysis for eNOS protein in cerebral cortex microvessels, the basilar artery, and the aorta in nonalcohol-fed and alcohol-fed rats. There was no significant difference in eNOS protein in cerebral microvessels, the basilar artery, and the aorta in nonalcohol-fed and alcohol-fed rats (P > 0.05).

DISCUSSION

There are two major new findings in this study. First, treatment of the cerebral microcirculation with BH4 significantly ameliorated impaired reactivity of pial arterioles in alcohol-fed rats toward that observed in nonalcohol-fed rats. Second, eNOS protein of cere-
A number of cofactors are critically important for the formation of nitric oxide from L-arginine, including BH4. Many recent studies have begun to examine the role of BH4 in impaired responses of peripheral blood vessels during disease states. A number of studies have shown that treatment of diabetic human subjects (11) or diabetic animals (22, 24, 28) with BH4 ameliorates impaired NOS-dependent vasoreactivity of large peripheral blood vessels. In addition, recent studies have shown that BH4 can restore impaired NOS-dependent dilatation of coronary blood vessels in human subjects (17, 37) and pigs (37) with atherosclerosis. Furthermore, Tiefenbacher et al. (38) report that impaired NOS-dependent dilatation of porcine coronary arteries following ischemia-reperfusion-induced injury could be prevented by acute treatment with sepiapterin, which is intracellularly converted to BH4. Finally, Hingman et al. (13) found that impaired NOS-dependent dilatation of the saphenous vein from smokers could be reversed by treatment with BH4. Thus it appears that an alteration in BH4 can contribute to impaired responses of peripheral blood vessels in humans and animal models during many disease states.

The mechanism by which BH4 can restore impaired NOS-dependent reactivity of blood vessels during disease states may relate to the uncoupling of eNOS due to an insufficiency of, or impaired metabolism of, BH4. Many investigators have suggested that, under various conditions, activation of eNOS can produce both nitric oxide and superoxide anion (26, 31, 39, 40). In addition, it appears that the metabolism of BH4 may play a key role in the control of production of nitric oxide and superoxide anion (1, 27, 31, 39, 40, 43). Thus in the absence of, or impaired metabolism of, BH4 there is an uncoupling of the L-arginine-nitric oxide pathway, resulting in an increased formation of superoxide anion and a reduced formation of nitric oxide (3–5, 8, 40, 43).

In the present study, we examined the effects of treatment with BH4 on reactivity of cerebral arterioles during chronic alcohol consumption. We found that treatment of pial arterioles with BH4 could ameliorate impaired NOS-dependent vasoreactivity. Thus the findings of the present study complement that reported by others (5, 11, 13, 17, 22, 24, 28, 37, 38). The findings of the present study also extend that of previous studies by examining the effects of BH4 on cerebral resistance blood vessels in vivo and by examining the effects of BH4 during chronic alcohol consumption. Few studies have examined the effects of alcohol on important cofactors that may regulate the synthesis and/or release of nitric oxide in alcohol-fed rats. Thus it appeared that the formation of oxygen radicals, presumably superoxide anion, contributed to impaired NOS-dependent reactivity of cerebral blood vessels during alcohol consumption. We speculated that the formation of oxygen radicals, during activation with receptor-mediated agonists, inactivated nitric oxide and thereby impaired NOS-dependent vasodilatation. However, the precise pathway(s) involved in the receptor-mediated release of oxygen radicals could not be determined in our previous studies (32, 33).

In previous studies (32, 33), we found that treatment of cerebral blood vessels (pial arterioles and the basilar artery) with topical application of superoxide dismutase could restore, in part, impaired NOS-dependent vasodilatation in alcohol-fed rats. Thus it appeared that the formation of oxygen radicals, presumably superoxide anion, contributed to impaired NOS-dependent reactivity of cerebral blood vessels during alcohol consumption. We speculated that the formation of oxygen radicals, during activation with receptor-mediated agonists, inactivated nitric oxide and thereby impaired NOS-dependent vasodilatation. However, the precise pathway(s) involved in the receptor-mediated release of oxygen radicals could not be determined in our previous studies (32, 33).

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**Fig. 4.** Assessment of the effect of chronic alcohol consumption on endothelial nitric oxide synthase (eNOS) protein. A: representative Western immunoblot of eNOS protein in cerebral cortex (CX) microvessels (lane 1, Control; lane 2, Alcohol), basilar artery (lane 3, Control; lane 4, Alcohol), and aorta (lane 5, Control; lane 6, Alcohol). B: each indicates the quantified data (Control, n = 8; and Alcohol, n = 11). Optical density of eNOS-positive bands was quantified by scanning densitometry and plotted relative to control. Values are means ± SE.
BH4 could contribute to impaired reactivity of cerebral blood vessels during chronic alcohol consumption by favoring the production of superoxide anion over nitric oxide.

In addition to potential alterations in cofactors that may regulate the synthesis and/or release of nitric oxide, it is possible that impaired responses of cerebral blood vessels during chronic alcohol consumption may be related to a direct effect of alcohol on the isoforms of NOS. Several studies have investigated the effects of acute and chronic exposure to alcohol on various aspects (mRNA, protein, and activity) of the isoforms of NOS. Regarding neuronal NOS, it is possible that an increase in the activity of neuronal NOS may produce an increase in oxygen radicals (1) and may account for impaired responses of cerebral arterioles during alcohol exposure. However, findings regarding the effects of alcohol on neuronal NOS are not entirely clear. Ikeda et al. (15) report that acute or chronic exposure of mice to alcohol did not alter neuronal NOS activity or nitric oxide production in various brain regions. In contrast, Xia et al. (42) found that chronic exposure (2 mo) of rats to an alcohol-containing diet produced a significant increase in neuronal NOS activity in the cerebellum. Finally, Fataccioli et al. (7) examined the effects of acute treatment with alcohol (25–200 mM) on rat cerebellar NOS activity. These investigators (7) report that acute exposure to alcohol inactivated neuronal NOS in a dose-dependent manner. Furthermore, the inactivation of neuronal NOS by alcohol could be prevented by treatment with BH4. These investigators (7) suggested that alcohol may reduce NOS activity by a modulation in the conformation of the enzyme to affect its stability, probably by interacting with the binding sites for BH4. Thus several studies have examined the effects of alcohol on neuronal NOS. The discrepancy between these studies regarding the effect of alcohol on neuronal NOS is not entirely clear, and it remains uncertain as to whether increases and/or decreases in neuronal NOS could account for impaired responses of cerebral arterioles during chronic alcohol consumption.

Studies have shown that stimulation of the inducible isoform of NOS can impair endothelium-dependent dilatation of blood vessels (14, 25). Thus it is conceivable that an increase in inducible NOS could account for impaired responses of cerebral arterioles during chronic alcohol exposure. However, studies that have examined the effects of alcohol on inducible NOS have reported conflicting findings. Syapin (35, 36) has reported that acute and chronic exposure of rat glioma cells to ethanol produced a decrease in the activity of inducible NOS. In addition, Zhao et al. (45) report that acute treatment of rat lung alveolar macrophages in vitro with ethanol produced a decrease in inducible NOS mRNA and protein. In contrast, Banan et al. (2) report that acute treatment of intestinal cells with ethanol increases inducible NOS activity. No studies that we are aware of have examined the activity of inducible NOS in the cerebral cortex during chronic exposure to alcohol. However, previous results (35, 36) do not appear to support the concept that an increase in inducible NOS during chronic alcohol consumption could contribute to impaired NOS-dependent dilatation of cerebral arterioles.

Findings regarding the effects of alcohol on eNOS expression (mRNA), protein, and enzyme activity are also controversial. Slomiany et al. (29) report that rats fed an alcohol-containing diet for a period of ~3 wk exhibited a pronounced decrease in eNOS activity in the buccal mucosa. In contrast, other investigators have reported that acute exposure to alcohol produced an increase in eNOS mRNA, protein, and activity in human placental villous tissue (16) and in cultured bovine aortic endothelial cells (12, 41). However, no studies that we are aware of have examined eNOS protein, expression, and/or activity of cerebral blood vessels during chronic alcohol consumption. From our findings in previous studies (18, 32, 33), we considered the possibility that impaired responses of cerebral blood vessels during chronic alcohol consumption may be related to a direct effect of alcohol on eNOS and thus the synthesis and/or release of nitric oxide. However, in the present study we found that eNOS protein level in cerebral arterioles, the basilar artery, and the aorta was similar in nonalcohol-fed and alcohol-fed rats. Thus it appears that impaired responses of cerebral blood vessels during alcohol consumption may not be related to an alteration in the quantity of eNOS protein per se. We speculate that altered reactivity of cerebral blood vessels during chronic alcohol consumption may be related to an imbalance in the formation of nitric oxide versus superoxide anion by receptor-mediated activation of eNOS.

In summary, in the present study we examined two potential mechanisms that may contribute to impaired responses of cerebral arterioles during chronic alcohol consumption. First, we examined the role of an alteration in BH4. We found that acute treatment of the cerebral microcirculation with BH4 could ameliorate impaired NOS-dependent responses of cerebral arterioles in alcohol-fed rats. Second, we examined the possibility that chronic alcohol consumption directly altered the enzyme for nitric oxide production in cerebral arterioles, i.e., eNOS. We found that eNOS protein was similar in cerebral blood vessels in nonalcohol-fed and alcohol-fed rats. From these findings, we suggest that impaired NOS-dependent responses of cerebral blood vessels during alcohol consumption may be related to an absence and/or an impaired metabolism of BH4 to uncouple eNOS. The uncoupling of eNOS in the endothelium of cerebral blood vessels may lead to endothelial dysfunction in alcohol-fed rats by a diminished production of nitric oxide and/or an increased production of superoxide anion to inactivate nitric oxide.

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