Exercise training restores impaired dilator responses of cerebral arterioles during chronic exposure to nicotine

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Mayhan WG, Arrick DM, Sun H, Patel KP. Exercise training restores impaired dilator responses of cerebral arterioles during chronic exposure to nicotine. J Appl Physiol 109: 1109–1114, 2010. First published August 12, 2010; doi:10.1152/japplphysiol.00564.2010.—Our goal was to determine whether exercise training (ExT) alleviates impaired nitric oxide synthase (NOS)-dependent dilation of pial arterioles during chronic exposure to nicotine. We measured dilation of cerebral (pial) arterioles in sedentary and exercised control and nicotine-treated (2 mg·kg⁻¹·day⁻¹ for 4 wk via an osmotic minipump) rats to an endothelial NOS (eNOS)-dependent (ADP), a neuronal NOS (nNOS)-dependent [N-methyl-D-aspartic acid (NMDA)], and a NOS-independent (nitroglycerin) agonist. In addition, we harvested brain tissue from sedentary and exercised control and nicotine-treated rats to measure the production of superoxide anion and measured superoxide dismutase-1 (SOD-1) protein in cerebral microvessels using Western blot. We found that eNOS- and nNOS-dependent, but not NOS-independent, vasodilation was impaired in nicotine-treated compared with control rats. In addition, the production of superoxide anion (lucigenin chemiluminescence) was increased, and SOD-1 protein decreased, in rats treated with nicotine compared with control rats. Further, although ExT did not significantly affect eNOS- or nNOS-dependent vasodilation in control rats, ExT restored impaired eNOS- and nNOS-dependent responses in nicotine-treated rats. In addition, the increase in superoxide anion production observed in nicotine-treated rats was reduced by ExT, and SOD-1 protein was increased in nicotine-treated rats by ExT. We suggest that ExT restores impaired NOS-dependent dilation of pial arterioles during chronic exposure to nicotine by a mechanism related to the formation of superoxide anion.

CIGARETTE SMOKING and/or the use of smokeless tobacco products leads to vascular damage in several major organ systems, including the lung, heart, and brain (1, 24, 29). In addition, investigators have shown that cigarette smoking and/or the use of smokeless tobacco contributes to the pathogenesis of ischemic and hemorrhagic stroke (4, 7, 21). Although cigarette smoke contains many toxic substances that could potentially damage cells composing the vasculature and surrounding tissue, it has been suggested that nicotine may be a contributing agent to vascular dysfunction during exposure to cigarette smoke and/or smokeless tobacco. Previous studies have shown that nicotine is toxic to endothelium (27, 33) and exposure (acute and chronic) to nicotine impairs nitric oxide synthase (NOS)-dependent dilation of large (50, 52) and small (28, 39, 40) peripheral vessels and cerebral vessels (13, 14, 19, 37). The mechanism by which nicotine impairs NOS-dependent dilation of large and small peripheral and cerebral blood vessels appears to be related to an increase in oxidative stress (6).

The beneficial effects of exercise training (ExT) on the prevention of cardiovascular-related diseases have been well-documented. Although the precise mechanism accounting for the beneficial effects of ExT on the cardiovascular system remains uncertain, several studies have suggested that ExT may modulate vascular endothelial function. Investigators have shown that ExT enhances eNOS-dependent dilation of skeletal muscle and cutaneous vessels in animals and humans via a change in shear stress to increase eNOS activity and/or via an increase in activity of antioxidant enzymes (17, 32, 51, 53, 63). In addition, ExT improves responses of peripheral and cerebral vessels during chronic disease states (3, 10, 25, 38, 44, 56). However, no studies to our knowledge have examined the influence of ExT on responses of cerebral arterioles during exposure to nicotine. Thus the goal of the present study was to examine the influence of ExT on reactivity of cerebral arterioles during chronic exposure to nicotine and determine a possible mechanism for the influence of ExT on reactivity of cerebral arterioles. To accomplish this goal, we measured eNOS- and nNOS-dependent reactivity of pial arterioles, superoxide anion production by brain tissue, and superoxide dismutase-1 (SOD-1) protein in sedentary and exercised control and nicotine-treated rats.

MATERIALS AND METHODS

Preparation of animals. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center, and all studies conform to the American Physiological Society’s “Guiding Principles in the Care and Use of Animals.” Adult male Sprague-Dawley rats (250–300 g) were divided into saline-treated (control) and nicotine-treated groups. In both groups, an osmotic minipump (model 2006; Alzet; Cupertino, CA) was implanted subcutaneously under anesthesia (pentobarbital sodium; 35–50 mg/kg ip). In the control group, the minipump contained vehicle (saline) and in the nicotine group the minipump contained nicotine at a concentration of 175 mg/ml. The minipump released saline or nicotine at a rate of 0.15 µl/h, to provide a concentration of nicotine at ~2 mg·kg⁻¹·day⁻¹, similar to that described previously (15, 19, 36). Four weeks after implantation of the minipump, rats were anesthetized with thiobutabarbital sodium (Inactin; 100 mg/kg body wt ip). A tracheotomy was performed and the animals were mechanically ventilated. A catheter was placed in a femoral vein for injection of supplemental anesthetic (10–20 mg/kg), and a femoral artery was cannulated to measure arterial pressure. After these procedures, a window was prepared over the parietal cortex to expose the pial microcirculation (14). The cranial window was suffused with artificial cerebral spinal fluid bubbled with 95% nitrogen-5% carbon dioxide. 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 into the suffusate. Arterial blood gases were monitored and maintained within normal limits.
Exercise training. Rats were exercised using standard techniques similar to what we (38) and others have described (10, 26, 46, 53, 63). Treadmill exercise was started 3 days following implantation of minipumps and was carried out 5 days/wk until the day before the experiment (4 wk after implantation of the minipumps). The length of time on the treadmill was initially 10 min/day for the first 3 days at 0% grade and a speed of 15–20 m/min. Then, over a 7-day period, the speed was increased to 25 m/min, the grade was increased to 10%, and the duration on the treadmill was increased to 60 min. This regimen for ExT is considered moderate and results in about a 45% increase in citrate synthase activity in the soleus muscle of rats (38) and is similar to that reported (23, 59).

Pial arteriolar diameter. Inner diameter of pial arterioles was measured using a video image-shearing device coupled to a video monitor. Diameter of arterioles was measured before, at 1-min intervals for 5 min during application of agonists, and after application of agonists was completed.

Experimental protocol. The cranial window was suffused for 30–45 min before testing responses to the agonists. In the first group (n = 5), we examined the effects of chronic treatment with nicotine on reactivity of pial arterioles in sedentary rats to an eNOS-dependent agonist [ADP (10 and 100 μM)], to an nNOS-dependent agonist [N-methyl-D-aspartic acid (NMDA; 100 and 300 μM)], and to a NOS-independent agonist [nitroglycerin (1.0 and 10 μM)]. In a second group (n = 7), we examined responses of pial arterioles to the agonists in exercised rats chronically treated with nicotine. In a third group (n = 7), we examined responses of arterioles to the agonists in sedentary control rats. In a fourth group (n = 8), we examined responses of pial arterioles to the agonists in exercised control rats.

Measurement of superoxide. Superoxide levels were measured by lucigenin-enhanced chemiluminescence as described previously (11, 12, 42). In separate groups of sedentary and exercised control (n = 11) and nicotine-treated (n = 8) rats, the brain was removed and placed in a Krebs/HEPES buffer (pH 7.4) with the following composition (in mmol/l): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgCl₂, 1.3 CaCl₂, 10 HEPES, 25 NaHCO₃, and 5 glucose. Samples of cortex tissue, cut from brains of sedentary and exercised control and nicotine-treated rats, were placed in polypropylene tubes containing 5 μM lucigenin. The tubes were then read in a Sirius/FB15 luminometer (Bertol Detection Systems), which reports relative light units (RLU) emitted over a 30-s interval for 5 min. Levels of superoxide reported are the value of tissue plus lucigenin-containing buffer minus background (lucigenin-containing buffer without tissue) and are normalized for tissue weight (RLU·min⁻¹·mg tissue⁻¹). We measured levels of superoxide in tissue obtained from sedentary and exercised control and nicotine-treated rats under basal conditions.

Western blot analysis. In separate groups of sedentary and exercised control and nicotine-treated rats (n = 12 for all groups) cerebral microvessels were isolated from brain tissue using methods described previously (62). Samples were homogenized in 20% (wt/vol) ice-cold buffer containing 10 mM Tris·HCl (pH 7.4), 1% SDS, 1 mM sodium vanadate, 10 μg/ml aprotinin, 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 concentrations in supernatant were determined by the Bradford method (Bio-Rad; Richmond, CA) with BSA as the standard. Protein was mixed and boiled in SDS-PAGE sample buffer for 5 min, then loaded and run on standard 7.5% or 12.5% gels using 20 μg of protein. After SDS-PAGE, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. Immunoblotting was performed with rabbit anti-SOD-1 (Santa Cruz Biotechnology) as the primary antibody and goat anti-rabbit as the secondary antibody. The bound antibody was detected using an ECL kit and quantified by scanning densitometry.

Drugs. ADP, lucigenin, and nicotine were purchased from Sigma Chemical (St. Louis, MO). Nitroglycerin was purchased from SoloPak Laboratories (Elk Grove Village, IL).

Statistical analysis. ANOVA with Scheffé’s test was used to compare responses of pial arterioles to the agonists and superoxide production in sedentary and exercised control and nicotine-treated rats. A P value of 0.05 or less was considered to be significant.

RESULTS

Baseline parameters. We found that baseline diameter of pial arterioles was similar in sedentary and exercised control and in exercised nicotine-treated rats (Fig. 1). In addition, there was no significant difference in blood pressure between sedentary and exercised control and nicotine-treated rats (Fig. 1).

Reactivity of pial arterioles. First, we determined the effect of ExT on responses of pial arterioles in control rats. We found that application of ADP (Fig. 2), NMDA (Fig. 3) and nitroglycerin (Fig. 4) dilated pial arterioles in sedentary and exercised control rats, and the magnitude of this dilation was similar in sedentary and exercised control rats.

Next, we examined the influence of ExT on responses of pial arterioles in rats treated with nicotine. We found that dilation of pial arterioles to ADP (Fig. 2) and NMDA (Fig. 3) was impaired in sedentary rats treated with nicotine compared with sedentary and exercised control rats. However, dilation of pial arterioles in response to nitroglycerin was not altered in sedentary rats treated with nicotine (Fig. 4). In addition, we found that ExT restored impaired responses of pial arterioles to ADP (Fig. 2) and NMDA (Fig. 3) in rats treated with nicotine.

![Fig. 1. Baseline diameter of pial arterioles and mean arterial pressure in sedentary and exercised control and nicotine-treated rats. Values are means ± SE. ExT, exercise training.](http://jap.physiology.org/ Downloaded from)
However, ExT did not influence reactivity of pial arterioles to nitroglycerin (Fig. 4).

**Superoxide production.** We measured superoxide production by cortex tissue in the various groups of rats. We found that the basal production of superoxide from cortex tissue was similar in sedentary and exercised control rats (Fig. 5). In addition, we found that basal production of superoxide from cortex tissue was increased in rats chronically treated with nicotine. Further, the increase in basal production of superoxide in the nicotine-treated rats was restored to that observed in control rats by ExT (Fig. 5). Thus it appears that ExT can reduce basal increases in superoxide production by cortex tissue in rats chronically treated with nicotine.

**Western blot analysis.** Chronic administration of nicotine produced a decrease in the expression of SOD-1 (Fig. 6). In addition, while ExT did not alter SOD-1 protein expression in control rats, ExT produced an increase in SOD-1 protein expression in rats treated with nicotine.

**DISCUSSION**

This is the first study that we are aware of that has examined the beneficial effects of ExT on reactivity of cerebral blood vessels during chronic exposure to nicotine. There are three new findings of the present study. First, we found that ExT alleviates impaired eNOS-dependent dilation of pial arterioles in nicotine-treated rats. Second, we found that ExT alleviates impaired nNOS-dependent dilation of pial arterioles in rats treated with nicotine. This effect of ExT on eNOS- and nNOS-dependent vasodilation appears to be related to NOS activity and not altered NO reactivity since ExT did not alter dilation of pial arterioles in response to nitroglycerin. Third, it appears that ExT may restore impaired eNOS- and nNOS-dependent dilation of pial arterioles by a mechanism that involves the inhibition of superoxide anion production. We found that superoxide production by cortex tissue was increased under basal conditions in rats treated with nicotine and this basal production was reduced by ExT.
increase in superoxide production was inhibited by ExT. Based on these findings, we suggest that ExT has beneficial effects on cerebral blood vessels and we speculate that ExT may have potential therapeutic value for the treatment of smoking-induced vascular dysfunction.

Influence of smoking/nicotine on vascular function. Previous studies have reported that active and passive exposure to cigarette smoke/cigarette smoke extract impairs NOS-dependent reactivity of large and small peripheral (8, 41, 45, 60) and cerebral (57) vessels in animals and human subjects. Mechanisms by which cigarette smoking/cigarette smoke extract impairs NOS-dependent reactivity are not entirely clear, but it appears that an increase in the local/systemic formation of reactive oxygen species may play a role (16, 18, 22, 41, 45, 49). Over the past several years, it has become apparent that nicotine may be a candidate contributing to vascular dysfunction in smokers and users of tobacco products. Investigators have reported that treatment of human subjects (9, 55) and animals (43) with nicotine impaired NOS-dependent reactivity of peripheral vessels. In addition, we (13, 14, 37) and others (19) have shown that acute and chronic treatment with nicotine produced selective impairment in NOS-dependent reactivity of cerebral arteries and arterioles that was attributed to the formation of oxygen radicals, presumably superoxide. The results of the present study complement and extend previous findings. In the present study, we found that chronic exposure to nicotine impaired NOS-dependent responses of cerebral resistance arterioles. In addition, we found that ExT could restore this alteration in cerebrovascular function produced by chronic exposure to nicotine, and this was correlated with decreased SOD-1 content and increased brain superoxide levels.

One might suggest that it is not entirely clear whether changes in protein levels of SOD-1 and production of superoxide are mechanistically linked in improving/inhibiting dilation of cerebral arterioles, respectively. To address this con-cern, studies could be conducted using SOD knockout mice to determine whether nicotine and/or ExT restores impaired responses of arterioles when changes of SOD-1 protein are prevented. However, comparisons between rats and knockout mice may be problematic. Another approach could be to inhibit superoxide production and determine whether nicotine and/or ExT alter dilation of cerebral arterioles. In a previous study (13) we found that chronic treatment of rats with apocynin, to inhibit superoxide production via activation of NADPH oxidase, could prevent nicotine-induced impairment in dilation of cerebral arterioles. Thus, although we cannot exclude the possible involvement of other cellular networks in impairment of vascular function during exposure to nicotine, we suggest that are findings regarding SOD-1 and superoxide are significant and appear to be mechanistically linked to dilation of cerebral arterioles.

We did not observe an effect of nicotine treatment on resting diameter of cerebral arterioles. Given the dramatic effect of nicotine treatment on cerebrovascular function, it was somewhat surprising that chronic treatment with nicotine did not influence basal diameter of cerebral arterioles. One might have predicted that since nicotine treatment influenced NOS-dependent responses of cerebral arterioles that this treatment may have led to a decrease in baseline diameter of cerebral arterioles. Although we are not certain as to why treatment with nicotine did not influence baseline diameter, we suggest that since baseline tone of cerebral arterioles may be related to a complex interplay between dilator/constrictor pathways, it is possible that compensatory mechanisms contribute to maintenance of vascular tone during treatment with nicotine.

Effects of ExT on vascular function. Many studies have examined the effects of ExT on NOS-dependent reactivity (31, 32, 61, 63). Sun et al. (63) found that short-term (2–4 wk) daily ExT increased NOS-dependent, but not -independent, dilation of skeletal muscle arterioles in rats. In addition, Kvernmo et al. (32) found that NOS-dependent dilation of cutaneous vessels from human subjects was increased by physical conditioning. Further, Koller et al. (31) found that ExT increased flow-dependent dilation of skeletal muscle arterioles from rats. The precise mechanisms by which ExT enhances NOS-dependent relaxation of blood vessels are not entirely clear but have been reported to be related to an increase in shear forces to increase the release of nitric oxide from the endothelium (53, 63), an increase in the release of prostanoids (31), an increase in the activity of superoxide dismutase (35, 54) and/or an increase in the activity of other antioxidant enzymes (glutathione peroxidase and catalase) (51). However, not all studies have shown an affect of ExT on NOS-dependent reactivity of blood vessels. Franke et al. (17) found that while ExT enhanced forearm vascular conductance in humans, it did not increase vascular responsiveness to NOS-dependent agonists. Oltman et al. (48) found that ExT did not influence NOS-dependent responses of porcine coronary arteries. Further, we (38) have shown that ExT does not alter responses of the basilar artery in rats to an eNOS-dependent agonist (acetylcholine). In the present study, we did not find a difference in reactivity of pial arterioles to ADP or NMDA between sedentary and ExT control rats.

While many studies have examined the influence of ExT on reactivity of blood vessels during physiological states, others have examined the effects of ExT during pathophysiological states. Although no studies to our knowledge have examined

Fig. 6. Superoxide dismutase-1 (SOD-1) protein from cerebral microvessels in sedentary and exercised control and nicotine-treated rats. Top: a representative Western immunoblot of SOD-1 protein and GAPDH protein. Bottom: quantified data from Western blots. Values are means ± SE. *P < 0.05 vs. sedentary and exercised control rats, and exercised nicotine-treated rats. **P < 0.05 vs. sedentary nicotine-treated rats.
the effects of ExT on responses of cerebral arterioles during chronic exposure to nicotine, others have reported beneficial effects of ExT on responses of peripheral blood vessels during chronic hypertension (3, 25), heart failure (20, 30), and Type 2 diabetes (38, 44). In addition, we (38) have shown that ExT restores impaired responses of the basilar artery during Type 1 diabetes. The mechanism for the effects of ExT on improving NOS-dependent responses of blood vessels during disease states is not entirely clear but may be related to a decrease in oxidative stress and/or an increase in nitric oxide synthesis/release (3, 25, 34, 38, 47). In the present study, we found that ExT restored impaired eNOS- and nNOS-dependent dilation of pial arterioles in rats chronically treated with nicotine. This restoration in NOS-dependent reactivity appeared to be related to an influence of ExT on oxidative stress since ExT reduced basal production of superoxide.

Implications of ExT during smoking/exposure to nicotine. Some may suggest that ExT may not be appropriate and/or feasible in smokers or users of tobacco products. However, there are many examples in the literature that suggest that ExT is tolerated by smokers and users of tobacco products and can reduce the risk of many diseases by a mechanism that may be related to a reduction in oxidative stress. A recent study by Anton et al. (2) found that ExT (8 h/wk) in individuals that smoked between 8–10 cigarettes/day for at least 2 years could be easily tolerated and that ExT reduced the risk for cardiovascular-related diseases in smokers. In addition, a study by Sinner et al. (58) found that the risk of lung cancer was decreased by physical activity in women that smoke. Further, it has been reported that ExT in smokers can lower lipid peroxidation, suggesting that the beneficial effects of ExT during smoking may be related to a reduction in oxidative stress (5). Thus we suggest that ExT is an appropriate and feasible methodology for the treatment of vascular dysfunction in smokers or users of nicotine-containing tobacco products.

In summary, this is the first study that we are aware of to examine the effects of ExT on eNOS- and nNOS-dependent reactivity of cerebral resistance arterioles. We found that ExT alleviated impaired eNOS- and nNOS-dependent dilation of pial arterioles in rats treated with nicotine but did not alter NO-dependent vasodilation. In addition, we found that nicotine produced an increase in superoxide formation from brain tissue and that ExT alleviated nicotine-induced superoxide formation. On the basis of our findings, we suggest ExT improves NOS-dependent responses of pial arterioles during chronic exposure to nicotine by a mechanism that appears to suppress the formation of superoxide. We speculate that our findings may have important implications for the pathogenesis of cerebrovascular abnormalities, including stroke, observed in smokers and users of tobacco products.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES
NOCITINE AND CEREBROVASCULAR REACTIVITY


