Acute infusion of nicotine impairs nNOS-dependent reactivity of cerebral arterioles via an increase in oxidative stress

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Acute infusion of nicotine impairs nNOS-dependent reactivity of cerebral arterioles via an increase in oxidative stress. J Appl Physiol 103: 2062–2067, 2007. First published September 27, 2007; doi:10.1152/japplphysiol.00411.2007.—Our goals were to determine whether acute exposure to nicotine alters neuronal nitric oxide synthase (nNOS)-dependent reactivity of cerebral arterioles and to identify a potential role for oxidative stress in nicotine-induced impairment in nNOS-dependent responses of cerebral arterioles. We measured in vivo diameter of cerebral arterioles to nNOS-dependent (N-methyl-D-aspartate and kainate) and -independent (nitroglycerin) agonists before and during acute treatment with nicotine. We found that nNOS-dependent, but not -independent, vasodilatation was impaired during treatment with nicotine. In addition, treatment of the cerebral microcirculation with tempol (1 h before infusion of nicotine) prevented nicotine-induced impairment in nNOS-dependent vasodilatation. Furthermore, the production of superoxide anion (lucigenin prevented nicotine-induced impairment in cerebral arterioles. Dilatation of cerebral arterioles in response to NMDA and kainate also can be attenuated by specific inhibition of nNOS (7-nitroindazole) (17, 50). Activation of nNOS has been suggested to represent a mechanism responsible for the coupling of local cerebral metabolism to changes in cerebral blood flow (7, 15, 16, 19). Thus the release of nitric oxide via activation of nNOS represents a major pathway for the control of cerebral arteriolar diameter and cerebral blood flow. However, few studies have examined the influence of disease states on nNOS-mediated reactivity of cerebral blood vessels, and no studies that we are aware of have examined the influence of nicotine on nNOS-dependent reactivity of cerebral arterioles. The first goal of this study was to examine whether acute exposure to nicotine alters nNOS-dependent reactivity of cerebral arterioles. Given that oxidative stress is an important contributor to vascular dysfunction in a wide variety of disease states, our second goal was to examine whether oxidative stress contributed to impaired nNOS-dependent reactivity of cerebral arterioles during acute exposure to nicotine.

MATERIALS AND METHODS

Preparation of animals. Adult male Sprague-Dawley rats (280–350 g) were used in these studies. All rats were housed in an animal care facility at the University of Nebraska Medical Center that is approved by the American Association for the Accreditation of Laboratory Animal Care, and all protocols were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Rats were anesthetized with thiobutabarbital sodium (Inactin; 100 mg/kg body weight ip). A tracheotomy was performed, and the animals were mechanically ventilated with room air and supplemental oxygen. A catheter was placed in a femoral vein for infusion of supplemental anesthetic (10–20 mg/kg, as necessary), for infusion of nicotine (2 μg·kg⁻¹·min⁻¹ for 30 min followed by a maintenance dose of 0.35 μg/kg for 1 min for the duration of the experiment), and for infusion of saline. This protocol, which our laboratory has used previously (30, 32), produces a plasma levels of nicotine similar to that seen in chronic smokers (10–40 ng/ml) (4, 5, 42, 45). A femoral artery was cannulated to measure arterial blood pressure. After these procedures, a window was prepared over the parietal cortex to expose the cerebral (pial) microcirculation (13). The cranial window was suffused with artificial cerebral spinal fluid bubbled with 95% nitrogen and 5% carbon dioxide. Temperature of the suffusate was maintained at 37 ± 1°C. The

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cranial window was connected via a three-way valve to an infusion pump, which allowed for infusion of agonists into the sulfamate. Arterial blood gases were monitored and maintained within normal limits.

**Cerebral arteriolar diameter.** The inner diameter of cerebral 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 5–10 min during application of agonists, and after application of agonists was completed. Application of vehicle did not alter diameter of cerebral arterioles. Baseline diameter of cerebral arterioles returned to control levels (before application of agonists) within 2–3 min after application of agonists was stopped.

**Experimental protocol.** The cranial window was suffused for 30 min before testing responses to the agonists. In the first group of rats (n = 5), we examined the effects of acute treatment with nicotine on reactivity of cerebral arterioles to nNOS-dependent agonists [NMDA (100 and 300 μM) and kainate (100 and 300 μM)] and to a nNOS-independent agonist [nitroglycerin (1.0 and 10 μM)]. After the rat was exsanguinated, the brain samples from the parietal cortex were placed in polypropylene tubes containing (in mmol/l): 118 NaCl, 4.7 KCl, 1.3 CaCl2, 1.2 MgCl2, 1.2 KH2PO4, 25 NaHCO3, 10 HEPES, and 5 glucose (pH 7.4). Tissue chemiluminescence (2, 29). After the rat was exsanguinated, the brain was removed and immersed in a modified Krebs-HEPES buffer containing (in mmol/l): 118 NaCl, 4.7 KCl, 1.3 CaCl2, 1.2 MgCl2, 1.2 KH2PO4, 25 NaHCO3, 10 HEPES, and 5 glucose (pH 7.4). Tissue samples from the parietal cortex were placed in polypropylene tubes containing 5 μmol/l lucigenin, and then they were read in a Fentomaster FB12 (Zytox) luminometer, which reports relative light units emitted integrated over 30-s intervals for 5 min. Data were corrected for background activity and normalized to tissue weight. In these studies, we measured superoxide anion production under basal conditions, during exposure to nicotine (20 ng/ml) for 30 min, and during exposure to nicotine in the presence of tempol.

**Statistical analysis.** Because responses to nitroglycerin, NMDA, and kainate were similar in all groups before infusion of nicotine, before infusion of nicotine in the presence of tempol, and before infusion of vehicle in the presence of tempol these data were pooled. Analysis of variance with Fischer’s test for significance was used to compare functional responses of cerebral arterioles between groups of rats, and superoxide anion production during treatment with nicotine in the absence and presence of tempol. A P value of ≤0.05 was considered to be significant.

**RESULTS**

**Reactivity of cerebral arterioles.** Application of NMDA (Fig. 1), kainate (Fig. 2), and nitroglycerin (Fig. 3) produced dose-related dilatation of cerebral arterioles. Acute infusion of nicotine did not alter baseline diameter of cerebral arterioles (50 ± 5 μm before infusion of nicotine vs. 51 ± 5 μm during infusion of nicotine; P > 0.05), mean arterial blood pressure (115 ± 5 mmHg before infusion of nicotine vs. 114 ± 5 mmHg during infusion of nicotine; P > 0.05), or heart rate (272 ± 17 beats/min before infusion of nicotine vs. 260 ± 14 beats/min during infusion of nicotine; P > 0.05). However, acute infusion of nicotine impaired reactivity of cerebral arterioles to NMDA (Fig. 1) and kainate (Fig. 2) but not to nitroglycerin (Fig. 3).

To determine whether infusion of nicotine impaired responses of cerebral arterioles via an increase in oxidative stress, we examined the influence of tempol on cerebrovascular reactivity during infusion of nicotine. Topical application of tempol to cerebral arterioles did not affect baseline diameter (48 ± 6 μm before application of tempol vs. 49 ± 6 μm during application of tempol; P > 0.05). In addition, infusion of nicotine in the presence of tempol did not influence baseline diameter of cerebral arterioles (49 ± 6 μm before infusion of nicotine vs. 49 ± 6 μm during infusion of nicotine in the presence of tempol; P > 0.05), mean arterial pressure (109 ± 7 mmHg before infusion of nicotine vs. 106 ± 8 mmHg during infusion of nicotine; P > 0.05), or heart rate (304 ± 26 beats/min before infusion of nicotine vs. 291 ± 33 beats/min during infusion of nicotine; P > 0.05). Furthermore, we found that treatment with tempol could prevent nicotine-induced impairment in reactivity of cerebral arterioles to NMDA (Fig. 1) and kainate (Fig. 2). However, treatment with tempol did not influence reactivity of cerebral arterioles to nitroglycerin (Fig. 3).

To determine whether there were any nonspecific effects of tempol on reactivity of cerebral arterioles, we examined
whether treatment with tempol could influence reactivity of cerebral arterioles to the agonists in the absence of infusion of nicotine. We found that topical application of tempol did not influence baseline diameter of cerebral arterioles (40 ± 3 μm before application of tempol vs. 40 ± 3 μm during application of tempol; P > 0.05). In addition, reactivity of cerebral arterioles to NMDA (Fig. 1), kainate (Fig. 2), and nitroglycerin (Fig. 3) were similar before and during treatment with tempol in the absence of infusion of nicotine. Thus it appears that treatment with tempol does not influence the reproducibility in responsiveness to the agonists.

Superoxide anion production. Basal production of superoxide anion increased dramatically when parietal cortex tissue was exposed to nicotine for 30 min (Fig. 4). Treatment of parietal cortex tissue with tempol did not influence basal superoxide anion production, but treatment with tempol significantly decreased nicotine-induced superoxide anion production by parietal cortex tissue (Fig. 4).

DISCUSSION

There are three new findings in this study. First, acute treatment with nicotine specifically impairs nNOS-dependent dilatation of cerebral arterioles. Second, treatment with tempol could prevent nicotine-induced impairment in nNOS-dependent dilatation of cerebral arterioles. Third, superoxide anion production from parietal cortex tissue was increased by treatment with nicotine, and tempol could inhibit this nicotine-induced increase in superoxide anion production. Based on these findings, we suggest that nicotine impairs nNOS-dependent dilatation of cerebral arterioles via a mechanism that involves an increase in the production of superoxide anion.

Consideration of methods. We used NMDA and kainate to examine nNOS-dependent responses of cerebral arterioles. Glutamate can stimulate neurons by activating several receptor subtypes, including NMDA and kainate. Activation of these receptors (NMDA family and non-NMDA receptors) are capable of stimulating the synthesis/release of neuronal nitric oxide (1, 20, 21, 37). Our laboratory (50) and others (7, 15–17) have shown that topical application of NMDA and kainate dilate cerebral arterioles. This vasodilatation appears to be related to the synthesis/release of nitric oxide (15, 16), presumably via activation of nNOS (17, 50). Thus it appears that the use of NMDA and kainate to evaluate nNOS-dependent dilatation of cerebral arterioles is appropriate. The findings from the present study suggest that nicotine impairs the nNOS component of dilatation of cerebral arterioles in response to NMDA and kainate. Because we did not measure neuronal activation (evoked potential) in the present study, we must assume that nicotine does not affect neuronal activation to NMDA or kainate.

We used tempol to examine a role of oxidative stress in impaired responses of cerebral arterioles during acute infusion of nicotine. Tempol is a membrane-permeable, biologically stable superoxide dismutase mimetic that has been used by many investigators to quench superoxide anion. The concentr-
traction of tempol used in the present study is similar to that reported by others (40, 41, 54). We found that tempol could restore nicotine-induced impairment in cerebrovascular reactivity and that tempol could prevent increases in superoxide anion formation by parietal cortex tissue in response to treatment with nicotine. In addition, we found that the effects of tempol were specific for nNOS-dependent responses because tempol did not influence vasodilatation to nitroglycerin. Furthermore, we found that tempol did not produce nonspecific effects on reactivity of cerebral arterioles since reactivity to the agonists was not influenced by tempol in the absence of infusion with nicotine. Thus it appears that tempol, at the concentration used in the present study, is efficacious and specific.

We measured the production of superoxide anion by parietal cortex tissue using lucigenin-derived chemiluminescence, as our laboratory (2) and others (10, 11, 27) have described previously. We found that exposure to nicotine increased superoxide anion generation by parietal cortex tissue. Superoxide anion production can occur from many cell types, including endothelium, vascular smooth muscle, neurons, and glia. In the present study, we cannot determine the precise cellular source of superoxide anion. It is conceivable that exposure to nicotine may actually stimulate an increase the production of superoxide anion from more than one cellular source. Thus it would be very difficult to determine, even using cell culture experiments, the overall importance of individual sources of superoxide anion formation by the various cell types in relation to altered vascular function. However, our inability to determine the precise source of superoxide anion does not diminish the importance of the finding that nicotine can stimulate the generation of superoxide anion, and this generation of superoxide anion appears to directly contribute to cerebrovascular dysfunction.

Consideration of previous studies. Several previous studies have reported that active and passive exposure to cigarette smoke/cigarette smoke extract impairs eNOS-dependent reactivity of large and small peripheral vessels in animals (34, 36, 44) and humans (8, 48) via the production of oxygen-derived free radicals (34, 36, 38). Increasing evidence suggests that nicotine may be a prime candidate contributing to vascular dysfunction in smokers and users of tobacco products. Investigators have reported that treatment of human subjects (9, 46) and animals (35) with nicotine impaired eNOS-dependent reactivity of peripheral vessels. In addition, our laboratory has reported that acute and chronic treatment of hamsters with nicotine produced selective impairment in eNOS-dependent reactivity of peripheral arterioles that was attributed to the formation of oxygen radicals (31, 33). Furthermore, our laboratory’s recent studies (12, 13) report that acute and chronic treatment of rats with nicotine, at a concentration found in smokers and users of tobacco products (5, 47), selectively impairs eNOS-dependent responses of cerebral arterioles via an increase in superoxide anion production. Thus it appears that nicotine can impair eNOS-dependent responses of cerebral arterioles via an increase in oxidative stress.

In addition to the synthesis/release of nitric oxide by cerebral endothelium via activation of eNOS, nitric oxide can also be synthesized/released from neurons and glial cells (astrocytes, microglia, and oligodendrocytes) in the brain by the activation of nNOS. Activation of nNOS has been suggested to be responsible, or at least substantially contribute to the coupling of local cerebral metabolism to changes in cerebral blood flow (7, 15, 16, 19). Thus the activation of nNOS represents a major network for the control of cerebral arteriolar diameter and cerebral blood flow. Investigators have examined the influence of cigarette smoking/nicotine exposure on nNOS activity/expression. Wuraga et al. (51) found that a 15-day exposure to nicotine produced an increase in nNOS=positive neurons in the brain of rats. In contrast, Hasan et al. (22) report that prenatal exposure to cigarette smoke significantly decreases nNOS expression in the neonatal brain of rats. In addition, Xie et al. (53) found that long-term passive smoking decreased penile nNOS activity/content in rats. Finally, Wright et al. (52) report that short- and long-term exposure to cigarette smoke did not influence nNOS gene expression or protein levels in rat lung. Thus there does not appear to be any general agreement regarding the influence of cigarette smoking/nicotine on nNOS activity/expression. In the present study we examined the influence of short-term exposure to nicotine on reactivity of cerebral arterioles. We suggest that this short-term exposure was not of sufficient length to alter the expression of nNOS in brain tissue and thus cannot account for impaired responses of cerebral arterioles to NMDA and kainate.

Although studies have examined the influence of cigarette smoking/nicotine exposure on nNOS activity/expression, the present study is novel in that we examined the influence of nicotine exposure on nNOS-mediated responses of cerebral arterioles. Because the activation of nNOS represents a major network for the control of cerebral arteriolar diameter and cerebral blood flow, it is critical to examine how disease states may alter this important network. To our knowledge, the present study is the first to examine the acute effects of nicotine on nNOS-dependent reactivity of cerebral arterioles. We found that acute exposure to nicotine could impair nNOS-dependent responses of cerebral arterioles. In addition, we extended this finding by examining the role of oxidative stress in impaired responses of cerebral arterioles during exposure to nicotine. We found that tempol could restore impaired nNOS-dependent reactivity of cerebral arterioles during exposure to nicotine. Although we have shown that oxidative stress may contribute to impaired nNOS-dependent reactivity during exposure to alcohol (49), this is the first study to suggest that nicotine exposure can impair nNOS-dependent responses of cerebral arterioles via an increase in oxidative stress. Thus it appears that nicotine influences a major vasodilator network in the brain via an increase in oxidative stress.

In summary, we examined the acute effects of nicotine on nNOS-dependent reactivity of cerebral arterioles. We found that treatment of rats with nicotine significantly impaired reactivity of cerebral arterioles to NMDA and kainate but not to nitroglycerin. In addition, we found that inhibition of superoxide anion formation by treatment with tempol could prevent impaired nNOS-dependent reactivity of cerebral arterioles during treatment with nicotine. Furthermore, exposure of brain tissue to nicotine dramatically increased superoxide anion formation, and this increase in superoxide anion could be inhibited by treatment with tempol. We speculate that our findings may have important implications for the pathogenesis of cerebrovascular abnormalities, including ischemic stroke, observed in smokers and users of tobacco products.
REFERENCES


