Inhibition of NADPH oxidase improves impaired reactivity of pial arterioles during chronic exposure to nicotine

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Fang, Qin, Hong Sun, Denise M. Arrick, and William G. Mayhan. Inhibition of NADPH oxidase improves impaired reactivity of pial arterioles during chronic exposure to nicotine. J Appl Physiol 100: 631–636, 2006.—Our goals were to determine whether chronic exposure to nicotine alters nitric oxide synthase (NOS)-dependent reactivity of cerebral (pial) arterioles and to identify a potential role for NADPH oxidase in impaired NOS-dependent responses during chronic exposure to nicotine. We measured in vivo diameter of pial arterioles to NOS-dependent (acetylcholine and ADP) and -independent (nitroglycerin) agonists in saline-treated rats and rats chronically treated with nicotine (2 mg·kg⁻¹·day⁻¹ for 2 wk via an osmotic minipump). We found that NOS-dependent, but not -independent, vasodilatation was impaired in nicotine-treated compared with saline-treated rats. In addition, the production of superoxide anion (lucigenin chemiluminescence) was increased in rats treated with nicotine compared with saline-treated rats. Furthermore, using Western blot analysis, we found that chronic exposure to nicotine increased p47phox protein in the parietal cortex. Finally, we found that apocynin (40 mg·kg⁻¹·day⁻¹) in the drinking water to inhibit NADPH oxidase alleviated impaired NOS-dependent cerebral vasodilatation in nicotine-treated rats but did not alter NOS-dependent responses in saline-treated rats and did not alter NOS-independent reactivity in saline- or nicotine-treated rats. These findings suggest that chronic exposure to nicotine impairs NOS-dependent dilatation of pial arterioles by a mechanism that appears to be related to the formation of superoxide anion via activation of NADPH oxidase.

MATERIALS AND METHODS

Preparation of animals. Adult male Sprague-Dawley rats (280–350 g) were divided into saline-treated and nicotine-treated groups. In both groups, an osmotic minipump (model 2004, Alzet, Cupertino, CA) was implanted subcutaneously under anesthesia [50 mg/kg ip methohexitol sodium (Brevital) or 35–50 mg/kg ip pentobarbital sodium]. In the saline-treated group, the minipump contained vehicle (saline) and in the nicotine group the minipump contained nicotine at a concentration of 100 mg/ml. The minipump released saline or nicotine at a rate of 0.25 µl/h to provide a concentration of nicotine at ~2 mg·kg⁻¹·day⁻¹, similar to that described previously (19, 22, 36). Two weeks after implantation of the minipump, rats were anesthetized with thiobutabarbitual 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 and obtain a blood sample for plasma nicotine and cotinine. After these procedures, a window was prepared over the parietal cortex to expose the pial microcirculation (17). 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. At the end of the experiment, an arterial blood sample was collected, and plasma was prepared and frozen at −70°C until assayed. Nicotine and cotinine concentrations in plasma were determined by mass spectrometry (19, 37). We elected to examine the concentration of cotinine in these studies since it is a major metabolite of nicotine.

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 inter-

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vals for 5 min during application of agonists, and after application of agonists was completed.

Experimental protocol. The cranial window was suffused for 30 min before testing responses to the agonists. In the first group of rats (n = 7), we examined the effects of chronic treatment with nicotine on reactivity of pial arterioles to NOS-dependent agonists [acetylcholine (1.0 and 10 μM) and ADP (10 and 100 μM)] and to a NOS-independent agonist [nitroglycerin (1.0 and 10 μM)]. In a second group of rats (n = 5), the minipump contained saline instead of nicotine, and we measured responses to acetylcholine, ADP, and nitroglycerin, as described above. In a third group of rats (n = 6), we examined the role of NADPH oxidase in chronic nicotine-induced impairment in reactivity of pial arterioles to NOS-dependent agonists. In these studies, rats were treated with nicotine (2 mg·kg⁻¹·day⁻¹ via an osmotic minipump) and apocynin (40 mg·H₁₁₀₀₅ l/min/kg·day⁻¹ in the drinking water) for 2 wk, and then we examined responses to the agonists. In a fourth group (n = 6), we examined the effects of apocynin in control rats treated with saline only.

Isolation of pial arterioles and cortex tissue. In separate groups of saline- (n = 15) and nicotine-treated (n = 15) rats, individual pial arterioles, devoid of meningeal tissue, were dissected from brain tissue using a microscope. Pial arterioles from three rats were pooled to have an adequate sample size for the measurement of NADPH oxidase subunits (nox-1, nox-4, p22phox, p47phox, and p67phox). Cerebral cortex tissue also was harvested and used for the measurement of protein for the various subunits of NADPH oxidase (nox-1, nox-4, p22phox, p47phox, and p67phox). All samples were rinsed with PBS, frozen on dry ice, and stored at −80°C until Western blot analysis.

Western blot analysis. Parietal cortex tissue samples and pial arterioles 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 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 bovine serum albumin as the standard. Protein levels were determined using an enhanced chemiluminescence kit and quantified by antibody directed against the various subunits of NADPH oxidase (gp91phox, p67phox, p47phox, and p40phox). All samples were boiled in SDS-PAGE sample buffer for 5 min, then mixed and transferred onto a polyvinylidene difluoride membrane in 192 μl of transfer buffer (pH 7.4) with the following composition (in mM): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgCl₂, 1.3 CaCl₂, 10 HEPES, 25 NaHCO₃, and 10 glucose. Samples of cortex tissue, cut from brains of saline- and nicotine-treated rats, were placed in polypropylene tubes containing 5 μM lucigenin. The tubes were then read in a Sirius/FB15 luminometer (Berthold Detections Systems), which reports relative light units 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 (relative light units·min⁻¹·mg tissue⁻¹).

We measured levels of superoxide in tissue obtained from saline- and nicotine-treated rats under basal conditions and during acute (30 min) incubation with apocynin (100 μM).

Drugs. Acetylcholine, ADP, lucigenin, and nicotine were purchased from Sigma Chemical (St. Louis, MO). Nitroglycerin was purchased from SoloPak Laboratories (Elk Grove Village, IL). Apocynin was purchased from Calbiochem (San Diego, CA). Anti-gp91phox (sc-5827), p22phox (sc-20781), nox-1 (sc-5821), nox-4 (sc-21860), p67phox (sc-7663), p47phox (sc-7660), p40phox (sc-18252), IgG, and anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis. Comparisons between the two groups of rats were performed using unpaired t-tests. ANOVA with Scheffe’s test was used for multiple comparisons. A P value of ≤0.05 was considered to be significant.

RESULTS

Plasma nicotine and cotinine. Plasma nicotine and cotinine levels in the nicotine-treated rats were 10.4 ± 0.6 and 120 ± 65 ng/ml, respectively. These levels were significantly higher than those found in rats treated with saline, which had negligible nicotine and cotinine levels (<1 ng/ml).

Reactivity of pial arterioles. Baseline diameter of pial arterioles was 39 ± 4 μm in nicotine-treated rats and 40 ± 1 μm in saline-treated rats (P < 0.05). Application of acetylcholine, ADP, and nitroglycerin dilated pial arterioles in nicotine- and saline-treated rats (Fig. 1). However, the magnitude of vasodilation to acetylcholine and ADP was significantly less in rats treated with nicotine than in saline-treated rats (Fig. 1). For example, acetylcholine (1.0 and 10 μM) dilated arterioles by 7 ± 1 and 10 ± 2%, respectively, in saline-treated rats but only by 1 ± 1 and 3 ± 1%, respectively, in nicotine-treated rats (P < 0.05). ADP (10 and 100 μM) dilated arterioles by 10 ± 1 and 15 ± 1%, respectively, in saline-treated rats but only by 2 ± 1 and 3 ± 1%, respectively, in nicotine-treated rats (P < 0.05). In contrast, nicotine did not alter responses of pial arterioles to nitroglycerin (Fig. 1).

Effects of inhibition of NADPH oxidase. Baseline diameter of pial arterioles was 38 ± 3 μm in nicotine + apocynin-treated rats and 41 ± 1 μm in saline + apocynin-treated rats (P < 0.05). These values are similar to those observed in saline- and nicotine-treated rats that did not receive apocynin. Application of acetylcholine, ADP, and nitroglycerin dilated pial arterioles in saline + apocynin-treated and nicotine + apocynin-treated rats (Fig. 1). Compared with that observed in nicotine treated rats, impaired vasodilation to acetylcholine and ADP was alleviated in nicotine + apocynin-treated rats (Fig. 1). In addition, treatment of saline-treated rats with apocynin did not alter vasodilatation to acetylcholine, ADP, or nitroglycerin (Fig. 1).

NADPH oxidase subunit protein. To further examine a role for NADPH oxidase, we measured protein content in the various subunits of NADPH oxidase in pial cortex tissue and pial arterioles from saline- and nicotine-treated rats. We found that chronic treatment with nicotine did not alter protein levels of gp91phox, p67phox, or p40phox in pial cortex tissue but did significantly increase p47phox protein in pial cortex tissue (Fig. 2). In contrast, we found a similar level of protein for nox-1, nox-4,
p22phox, p47phox, or p67phox in pial arterioles isolated from saline- and nicotine-treated rats (Fig. 3).

**Superoxide production.** Basal production of superoxide was increased in brain tissue from rats chronically treated with nicotine compared with saline-treated rats (Fig. 4). In addition, acute incubation with apocynin did not alter superoxide production in tissue obtained from saline-treated rats but significantly decreased basal superoxide production in tissue obtained from rats chronically treated with nicotine (Fig. 4).

**DISCUSSION**

There are four new findings in this study. First, chronic treatment with nicotine impairs NOS-dependent, but not -independent, dilation of pial arterioles. Second, concomitant treatment of nicotine-treated rats with a selective inhibitor of NADPH oxidase (apocynin) alleviates impaired NOS-dependent vasodilation. Third, p47phox (a subunit of NADPH oxidase) protein was increased in the parietal cortex of rats.
chronically treated with nicotine. Fourth, superoxide produc-
tion from parietal cortex tissue was increased in rats chroni-
cally treated with nicotine, and apocynin inhibits this increase
in superoxide production. Based on these findings, we suggest
that chronic exposure to nicotine impairs NOS-dependent di-
latation of pial arterioles via a mechanism that appears to
involve an increase in the production of superoxide anion via
activation of NADPH oxidase.

Previous studies have reported that active and passive ex-
posure to cigarette smoke/cigarette smoke extract impairs
NOS-dependent reactivity of large and small peripheral vessels
in animals (40, 43) and humans (10, 53). In addition, investi-
gators have shown that cigarette smoking impairs reactivity of
cerebral vessels to hypercapnia (51). Mechanisms by which
cigarette smoking/cigarette smoke extract impairs reactivity are
not entirely clear, but it appears that an increase in the local/systemic formation of reactive oxygen
species may play a role. In studies involving animals, cigarette
smoke/cigarette smoke extract alters reactivity of large periph-
eral vessels via production of oxygen radicals (40, 43, 44), and
clinical studies have shown that treatment of smokers with
vitamin C protects against endothelial dysfunction (20, 21, 26).
However, cellular networks responsible for the formation of
oxygen radicals during exposure to cigarette smoke are not
entirely clear.

Increasing evidence appears to suggest that nicotine may be
a prime candidate for contributing to vascular dysfunction in
smokers and users of tobacco products. Investigators have
reported that treatment of human subjects (12, 49) and animals
(42) with nicotine impaired NOS-dependent reactivity of pe-
ripheral vessels. In addition, we have reported that acute and
chronic treatment of hamsters with nicotine produced selective
impairment in NOS-dependent reactivity of resistance arte-
rioles that was attributed to the formation of oxygen radicals
(38, 39). Furthermore, our recent study reports that acute
infusion of nicotine, at a concentration found in smokers,
selectively impaired NOS-dependent responses of pial arte-
rioles that appeared to be related to oxidative stress (17). The
results of the present study complement and extend our previ-
sous findings. In the present study, we treated rats with nicotine
for 2 wk and then examined responses of pial arterioles to
NOS-dependent and -independent agonists. Similar methodol-
dies have been used by others (19, 22, 36) to examine the
chronic effects of nicotine on the circulation. Plasma levels of
nicotine and cotinine measured in the present study are similar
to those reported by others (19, 22, 36) and are similar to those
reported in chronic smokers (6, 50). We found that chronic
treatment of rats with nicotine produced selective impairment
in NOS-dependent reactivity of pial arterioles. Thus it appears that nicotine may account for vascular dysfunction in smokers and users of smokeless tobacco products.

Because the formation of oxygen radicals appears to be important in vascular dysfunction in chronic smokers and during acute treatment with nicotine, we measured the production of superoxide using lucigenin-derived chemiluminescence, as described by others (13, 15, 32). We found that chronic treatment with nicotine increased levels of superoxide production by cortex tissue. Furthermore, treatment with apocynin significantly decreased superoxide production by brain tissue, suggesting that NADPH oxidase may be an important source of superoxide in rats chronically treated with nicotine. In addition to measuring superoxide production, we also conducted functional studies to examine the role of NADPH oxidase in impaired responses of pial arterioles to NOS-dependent agonists during chronic exposure to nicotine and biochemical studies to determine the influence of nicotine on various subunits for NADPH oxidase.

NAD(P)H oxidases are multi-component enzyme complexes that include two membrane-associated subunits (p22phox and gp91phox) and cytosolic subunits (p47phox, p67phox, and p40phox) (11). Assembly of these units also incorporates ATPase, Rac1, and Rac2 (11). It appears that phosphorylation of p47phox allows the association of cytosolic components with membrane-bound components to activate the enzyme complex utilizing NADPH as the electron donor for reduction of molecular oxygen to superoxide (16). A homolog of gp91phox, nox-1 (formerly nox-1), has been identified in rat aortic smooth muscle cells and endothelial cells of the basilar artery and appears to contribute to the production of reactive oxygen species (2, 35, 52). Another important homolog of gp91phox, nox-4, has been identified in endothelium and vascular smooth muscle of aorta and cerebral vessels (2, 3, 45). In fact, a recent study by Ago et al. (3) suggests that nox-4 may be an important catalytic component of endothelial NADPH oxidase. In the present study, we measured the influence of chronic exposure to nicotine on protein levels of various subunits of NADPH oxidase in cortex tissue and pial arterioles.

In the present study, we found that apocynin alleviated impaired NOS-dependent reactivity of pial arterioles in nicotine-treated rats. A number of recent studies have used apocynin to examine the effects of oxidative stress, via NADPH oxidase, on vascular dysfunction during a variety of disease states (7, 23, 24, 48). We also found that treatment with nicotine increased the protein expression of p47phox, but not gp91phox, p67phox, or p40phox, in cerebral cortex tissue but did not alter protein expression of nox-1, nox-4, p22phox, p47phox, or p67phox in pial arterioles. The finding that nicotine increased the expression of p47phox in parietal cortex tissue but did not alter the expression of any of the subunits for NADPH oxidase measured in pial arterioles was somewhat surprising. We speculate that this finding could suggest that an increase in nicotine-induced oxidative stress may be localized to structures other than those contained in pial arterioles, i.e., neurons and/or glia cells. Furthermore, it is possible that, although the expression of the subunits for NADPH oxidase is not increased in pial arterioles, the activity of the enzyme may be elevated in nicotine-treated rats compared with saline-treated rats. Although it was beyond the scope of the present study to examine enzyme activity, future studies will need to identify whether the activity of NADPH oxidase is increased in pial arterioles vs. parietal cortex tissue. Nevertheless, our findings are novel in that we report the damaging effects of chronic exposure to nicotine on reactivity of cerebral arterioles, the formation of superoxide during chronic exposure to nicotine, and the potential therapeutic effects of inhibition of NADPH on cerebrovascular dysfunction during exposure to nicotine.

In summary, we examined the role of NADPH oxidase in chronic nicotine-induced impairment of NOS-dependent reactivity of pial arterioles. We found that treatment of rats with nicotine impaired 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 cortex tissue in rats treated with nicotine. Furthermore, concomitant treatment with an inhibitor of NADPH oxidase alleviated nicotine-induced impairment of NOS-dependent reactivity. We suggest superoxide released via activation of NADPH oxidase contributes to impaired NOS-dependent dilation of pial arterioles during chronic exposure to nicotine. 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.

REFERENCES


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