Chronic nicotine exposure exacerbates transient focal cerebral ischemia-induced brain injury

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Li C, Sun H, Arrick DM, Mayhan WG. Chronic nicotine exposure exacerbates transient focal cerebral ischemia-induced brain injury. J Appl Physiol 120: 328–333, 2016. First published December 10, 2015; doi:10.1152/japplphysiol.00663.2015.—Tobacco smoking is a risk factor contributing to the development and progression of ischemic stroke. Among many chemicals in tobacco, nicotine may be a key contributor. We hypothesized that nicotine alters the balance between oxidant and antioxidant networks leading to an increase in brain injury following transient focal cerebral ischemia. Male Sprague-Dawley rats were treated with nicotine (2 or 4 mg·kg⁻¹·day⁻¹) for 4 wk via an implanted subcutaneous osmotic minipump and subjected to a 2-h middle cerebral artery occlusion (MCAO). Infarct size and neurological deficits were evaluated at 24 h of reperfusion. Superoxide levels were determined by lucigenin-enhanced chemiluminescence. Expression of oxidant and antioxidant proteins was measured using Western blot analysis. We found that chronic nicotine exposure significantly increased infarct size and worsened neurological deficits. In addition, nicotine significantly elevated superoxide levels of cerebral cortex under basal conditions. Transient focal cerebral ischemia produced an increase in superoxide levels of cerebral cortex in control group, but no further increase was found in the nicotine group. Furthermore, chronic nicotine exposure did not alter protein expression of NADPH oxidase but significantly decreased MnSOD and uncoupling protein-2 (UCP-2) in the cerebral cortex and cerebral arteries. Our findings suggest that nicotine-induced exacerbation in brain damage following transient focal cerebral ischemia may be related to a preexisting oxidative stress via decreasing of MnSOD and UCP-2.

Tobacco smoking is a serious social problem. It is an independent risk factor for cardiovascular disease and has a disadvantage for neurological disorders (9, 18). Smokers have a higher incidence of coronary artery disease, hypertension, peripheral vascular disease, and stroke (23). Furthermore, cardiovascular disease is the major cause of mortality in humans that smoke and/or use nicotine-containing tobacco products (31). An epidemiological study previously indicated that current or recent smokers experience poorer functional outcomes than nonsmokers after acute ischemic stroke (28). In contrast, a recent study found that tobacco smoking was independently associated with lower inpatient mortality in acute ischemic stroke (1). Although cigarette smoke contains many toxic substances, nicotine has been demonstrated as a key contributor to the adverse effects of tobacco products on the cardiovascular system and the brain (34). Acute treatment with nicotine has been shown to exacerbate brain damage following permanent and transient focal ischemia (5, 30), and this appeared to be associated with an increase in inflammatory markers. Due to the advances in intravascular techniques and thrombolytic agents, transient focal cerebral ischemia has become one of the most common types of stroke. Thus our first goal was to determine whether chronic treatment with nicotine dose dependently alters brain damage and neurological outcome following transient focal cerebral ischemia.

Although reperfusion is critical for restoring normal function following ischemic stroke, it can paradoxically result in secondary damage, called cerebral ischemia/reperfusion (I/R) injury. Oxidative stress is a major mechanism of cerebral I/R injury. Mitochondria are the main source of cellular reactive oxygen species (ROS) during reperfusion (17, 21). Mitochondrial ROS appear to exacerbate ROS production by NADPH oxidase (NOX) and from mitochondria (autostimulation) via a ROS-dependent ROS production mechanism in the vasculature (17, 42). Overproduction of mitochondrial ROS may contribute to cerebral I/R injury by intensifying inflammation (19, 32). In addition, mitochondrial ROS blocks mitochondrial respiration and facilitates mitochondrial transition pore formation, which may lead to the release of inner and outer mitochondrial membrane components including cytochrome c and apoptosis-inducing factor (7). Thus overproduction of ROS by mitochondria may play a pivotal role in activating apoptotic pathways following transient focal cerebral ischemia. MnSOD localizes in mitochondria and is of prime importance in maintaining cellular ROS balance and mitochondrial integrity (14, 37). A recent study found that genetic upregulation of MnSOD prevented inflammation and apoptosis following transient focal cerebral ischemia (15). Uncoupling protein-2 (UCP-2) is another mitochondrial protein negatively regulating mitochondrial ROS generation. Overexpression of UCP2 diminished cerebral I/R injury (26). Thus our second goal was to determine the influence of chronic nicotine exposure on superoxide levels and protein expression of NOX, MnSOD, and UCP-2 in the brain.

MATERIALS AND METHODS

Animal models of chronic nicotine exposure. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the Louisiana State University Health Science Center-Shreveport and conducted in accordance with the National Institutes of Health Guide for the Care and Use Laboratory Animals. Adult male Sprague-Dawley rats (250–300 g) were divided into control (n = 24) and nicotine groups (n = 33). An osmotic minipump (Alzet, Cupertino, CA) was implanted subcutaneously under anesthesia (sodium pentobarbital; 35–50 mg/kg ip) and aseptic conditions. The minipumps were filled with saline in the control groups and nicotine in nicotine groups. Nicotine was released at a rate of 2 or 4 mg·kg⁻¹·day⁻¹ via an implanted subcutaneous osmotic minipump. The animals were housed in individual cages and monitored daily for food and water intake and body weight. The rats were sacrificed 24 h after the final saline or nicotine exposure, and the brains were harvested for further analysis. The animals were killed by pentobarbital overdose.

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mg·kg\(^{-1}\)·day\(^{-1}\). Four weeks after implantation of the minipump, rats were subjected to cerebral I/R injury.

**Cerebral I/R injury.** Twenty-two rats (control group: \(n = 6\); 2 mg·kg\(^{-1}\)·day\(^{-1}\) nicotine group: \(n = 8\); 4 mg·kg\(^{-1}\)·day\(^{-1}\) nicotine group: \(n = 8\)) were subjected to cerebral I/R injury. Unilateral MCA occlusion (MCAO) was performed for 120 min as described previously (41). On the day of the experiment, rats were anesthetized with ketamine/xylazine (100/15 mg/kg ip). Rectal temperature was maintained at 37°C using a temperature controlled heating pad (Harvard Apparatus, March, Germany). A laser-Doppler flow probe (Perimed, Stockholm, Sweden) was attached to the right side of the dorsal surface of the skull (2 mm caudal and 5 mm lateral to the bregma) to monitor regional cerebral blood flow (rCBF). The right common and external carotid arteries were exposed and ligated. The middle cerebral artery (MCA) was occluded by inserting a monofilament suture (Doccol) into the internal carotid artery to the point where the MCA branched off from the internal carotid artery. Onset of the MCAO was determined by a rapid drop in rCBF. After the right MCA was occluded for 120 min, reperfusion was initiated by removing the suture. Animals were allowed to recover for 24 h. At 24 h of reperfusion, motor/sensory deficits were evaluated using a 24-point scoring system (13, 41). After neurological evaluation, the rats were euthanized with Inactin (150 mg/kg). The brain was quickly removed and placed in ice-cold saline for 5 min and cut into six 2 mm-thick coronal sections. Sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) for 15 min at 37°C. Slice images were digitalized, and the infarct lesion was evaluated using ImageJ. Complete lack of staining is defined as infarct lesion. Total lesion was expressed as percentage of the ipsilateral hemisphere (4).

**Superoxide measurement.** Nineteen rats with (control: \(n = 5\); nicotine: \(n = 4\)) or without (control: \(n = 5\); nicotine: \(n = 5\)) MCAO were used to measure to superoxide levels in cerebral cortex using lucigenin-enhanced chemiluminescence. Parietal cortex tissues were homogenized in 10% (wt/vol) ice-cold buffer containing 10 mmol/l Tris·HCl, pH 7.4, 0.32 M sucrose, 0.25 mM EDTA, and protease inhibitor cocktail. Homogenates were centrifuged at 12,000 g for 20 min at 4°C, the supernatants were used for superoxide measurement. Protein concentration in the supernatants was determined by the BCA method (Thermo Scientific) with BSA as the standard. The supernatants were added to ice-cold modified Krebs-HEPES buffer, which contained 5 μM lucigenin, and superoxide levels from the mixers were read using a luminometer (Berthold, Germany), which reports relative light units emitted integrated over 30-s intervals for 5 min.

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Effect of chronic nicotine exposure on brain injury following a 2-h middle cerebral artery occlusion (MCAO)/24-h reperfusion. A: representative 2,3,5-triphenyltetrazolium chloride staining. B: total infarct volume, cortical infarct volume, and subcortical infarct volume. C: neurological score. Values are means ± SE. *\(P < 0.05\) vs. control.
Data were corrected for background and normalized to protein concentration of the samples.

Western blot analysis. Parietal cortex and surface arteries of cerebral hemispheres were isolated from 16 rats (control: n = 8; nicotine: n = 8) for measuring protein expression of MnSOD, UCP-2, NOX-1, NOX-2, and NOX-4. The samples were homogenized in ice-cold lysis buffer containing 150 mmol/l NaCl, 50 mmol/l Tris·HCl, 10 mmol/l EDTA, 0.1% Tween-20, 1% Triton, 0.1% mercaptoethanol, 0.1 mmol/l phenylmethyl sulfonylfluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin, and 5 μg/ml aprotenin, pH 7.4. Homogenates were centrifuged at 4°C for 10 min at 10,000g, and the supernatants were collected. Protein concentration was determined by the Bradford method (Bio-Rad) with BSA as the standard. SDS-PAGE was performed on a 10% gel on which 20–30 μg of total protein per well was loaded. After SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride membrane. Immunoblotting was performed with the use goat anti-SOD-2 (Santa Cruz Biotechnology), mouse anti-UCP-2 (Santa Cruz Biotechnology), rabbit anti-NOX-1 (Novus Biologicals), goat anti-NOX-2 (Santa Cruz Biotechnology), and goat anti-NOX-4 (Santa Cruz Biotechnology) as primary and peroxidase conjugated mouse anti-rabbit and mouse anti-goat IgG as the second antibody. The bound antibody was detected by enhanced chemiluminescence (ECL) detection (Pierce Chemical), and the bands were analyzed using ChemiDoc MP Imaging System (Bio-Rad). For quantification, protein expression of MnSOD, UCP-2, NOX-1, NOX-2, and NOX-4 was normalized to the GAPDH.

Statistical analysis. Data are reported as means ± SE. Differences between groups were evaluated for statistical significance by ANOVA with Fisher’s test or by Student’s t-tests as appropriate. P ≤ 0.05 was considered to be significant.

RESULTS

Cerebral I/R injury. Changes in cerebral blood flow in response to MCAO were similar in the groups of rats. Blood flow decreased by 57 ± 5% in the control group, by 59 ± 5% in the 2 mg·kg⁻¹·day⁻¹ group and by 68 ± 3% in the 4 mg·kg⁻¹·day⁻¹ group (P > 0.05). However, there were significant increases in total lesion and cortical infarct volume in nicotine-exposed rats compared with the control rats (Fig. 1). In addition, the neurological deficit was significantly greater in nicotine-exposed rats. Interestingly, 4 mg·kg⁻¹·day⁻¹ nicotine exposure failed to further exacerbate 2-h MCAO/24-h reperfusion-induced brain damage and neurological deficit compared with 2 mg·kg⁻¹·day⁻¹ nicotine group. Because of this finding, we elected to use the 2 mg·kg⁻¹·day⁻¹ nicotine dose for the remainder of our experiments.

Superoxide levels. To determine whether oxidative stress plays a role in increased brain damage following transient focal cerebral ischemia in nicotine-exposed rats, we measured superoxide levels in the cerebral cortex. As shown in Fig. 2, superoxide levels under basal conditions and following cerebral I/R were significantly increased in 2 mg·kg⁻¹·day⁻¹ nicotine-exposed rats compared with the control rats. Cerebral I/R produced an increase in superoxide levels in the control group but failed to further increase superoxide levels in nicotine-exposed rats.

Protein expression of MnSOD, UCP-2, and NOXs. Since basal superoxide levels in the cerebral cortex were significantly enhanced in nicotine-exposed rats, we measured the protein expression of MnSOD, UCP-2, and NADPH oxidase isoforms, NOX-1, NOX-2, and NOX-4, in cerebral cortex and arteries. As shown in Figs. 3 and 4, protein expression of MnSOD and UCP-2 were significantly less in cerebral cortex of 2 mg·kg⁻¹·day⁻¹ nicotine-exposed rats. In addition, MnSOD was found to be less in cerebral arteries in the nicotine-treated rats (Fig. 3). In contrast, chronic nicotine exposure did not alter protein expression of NOXs in the cerebral cortex or arteries (Fig. 5).
DISCUSSION

The present study investigated the influence of nicotine on brain damage following transient focal cerebral ischemia. There are several new findings from this study. First, chronic nicotine exposure exacerbated brain damage following transient focal cerebral ischemia. Second, basal superoxide levels were increased in cerebral cortex and arteries during exposure to nicotine. Third, MnSOD and UCP-2 were significantly decreased in cerebral cortex and arteries of nicotine-exposed animals. Fourth, NOXs in cerebral cortex and arteries were not altered in nicotine-exposed animals. We speculate that tobacco smoking exacerbates cerebral I/R injury via a preexisting oxidative stress that may be related to a decreased expression of MnSOD and/or UCP-2.

Among many toxic substances, nicotine has been considered as a key component that contributes to the influence of cigarette smoke on the central nervous and cardiovascular systems. Nicotine can cross the blood-brain barrier and reach a high level in the brain within 10–20 s after inhalation (20). The subcutaneous osmotic minipump releases nicotine at a constant rate, which resulted in stable plasma nicotine and cotinine levels that match the chronic smokers. The doses of nicotine used in the present study have been reported to result in stable plasma nicotine levels corresponding reasonably well with plasma levels in habitual light (0.5–1 pack/day) and moderate (2 packs/day) smokers (25); thus we believe that our model is appropriate to investigate the chronic effects of nicotine tobacco smoking on the brain.

Several studies have investigated the influence of nicotine on I/R injury in heart, liver, kidney, and brain. Some studies have shown that acute exposure to nicotine was cytoprotective in these organs (6, 29, 33, 39). In contrast, others have shown that acute and chronic exposure to nicotine worsened renal and cerebral I/R injury. Arany et al. (3) found an exacerbated renal I/R injury in mice preexposed to nicotine for 4 wk. Wang et al. (38) reported that acute exposure (2 wk) to a high-dose of nicotine (4.5 mg·kg⁻¹·day⁻¹) increased 1-h MCAO/24-h reperfusion-induced infarction by 36% in rats. Recently, Bradford et al. (%) found that 30-min MCAO/3-day reperfusion-induced brain damage was worsened in mice exposed to low-dose nicotine for 2 wk. In the present study, we determined whether chronic nicotine exposure dose dependently alters cerebral I/R injury. Surprisingly, low-dose and high-dose nicotine exposures produced a similar increase in cerebral I/R injury. Thus habitual light smokers may be not expected to have better neurological prognosis than moderate and heavy smokers following transient focal cerebral ischemia.

A few studies have investigated the mechanism underlying the detrimental effect of chronic nicotine exposure on cerebral I/R injury. Wang et al. (38) suggested that the exacerbated cerebral I/R injury is associated with a depletion of brain microvascular tissue plasminogen activator (tPA) antigen. NOX-1 NOX-2 NOX-4 NOX-1 NOX-2 NOX-4

![Fig. 4. Effect of chronic nicotine exposure on protein expression of uncoupling protein-2 (UCP-2) in the cerebral cortex. Values are means ± SE. *P < 0.05 vs. control.](image)

![Fig. 5. Effect of chronic nicotine expression on protein expression of NADPH oxidase-1 (NOX-1), NOX-2, and NOX-4 in the cerebral cortex and arteries. Values are means ± SE.](image)
Bradford et al. (5) indicated that nicotine exposure aggravates the postischemic inflammatory response. In a recent study, Shah et al. (35) reported a reduced glucose transporter-1 (GLUT1) activity at the blood-brain barrier in mice preexposed with nicotine for 2 wk. The present study is the first to determine the potential role of oxidative stress on nicotine-induced cerebrovascular dysfunction. We found that superoxide levels in the cerebral cortex under basal conditions and following transient focal cerebral ischemia were significantly increased in rats preexposed to nicotine. Overproduction of superoxide not only directly poses a threat to cells by causing peroxidation of lipids, oxidation of proteins, and damage to nucleic acids but also indirectly contributes to cerebral I/R injury by intensifying postischemic inflammation and apoptosis (7, 19, 32). Recently, nicotine-induced oxidative stress has been found to be involved in renal I/R injury (2). In addition, cigarette smoking-induced reduction in the release of tPA antigen was restored by the antioxidant ascorbic acid (16). Furthermore, ROS downregulated GLUT1 in retinal endothelial cells (11). Antioxidant bilirubin upregulated the expression of GLUT1 expression/activity in vascular endothelial cells (8). Brain cells are especially vulnerable to ROS-mediated injury. The brain accounts for ~20% of the aerobic metabolism. Neurons are exposed to a minimum level of ROS from both exogenous and endogenous sources in normal conditions. However, the brain has a minimum storage capacity for oxygen and a high probability of lipid peroxidation. The content of both exogenous and endogenous antioxidants in the central nervous system is small compared with that of other tissues (40). Thus preexisting oxidative stress may be a critical factor contributing to the detrimental effect of chronic nicotine exposure on cerebral I/R injury.

Oxidative stress is essentially an imbalance between oxidants and antioxidants. NOX has been established as a major source for superoxide overproduction by tobacco smoking (18). During the last two decades, seven NOX isoforms have been identified in mammalian cells. Among these isoforms, NOX-1, NOX-2, and NOX-4 appear important in central nervous system and cardiovascular system (18, 27). In the present study, we did not detect an alteration in protein expression of NOX-1, NOX-2, and NOX-4 in the cerebral cortex and arteries of rats chronically exposed to nicotine. Interestingly, MnSOD activity of nicotine-exposed rats. However, it has to be noted that protein expression of NOXs does not always represent their functional activity, which is also regulated by the cytosolic subunits such as p47phox, p67phox, p40phox, and Rac. In a previous study, we found that chronic nicotine exposure upregulated p47phox expression in cerebral cortex and NOX inhibitor restored impaired reactivity of pial arterioles (10). Thus the possible role of NOX in chronic nicotine exposure-induced oxidative stress cannot be excluded in the present study. On the other hand, we found a reduced protein expression of MnSOD and UCP-2 in the cerebral cortex and arteries of nicotine-exposed rats. Interestingly, MnSOD activity of blood has been reported to be reduced in smokers compared with nonsmokers (24). In addition, chronic nicotine exposure was shown to reduce MnSOD activity in osteoblasts (22). Furthermore, UCP-2 level in peripheral blood lymphocytes from smokers was higher than that in nonsmokers (36). Mitochondria have been considered as the major source of cellular ROS in mammals under physiological conditions and during reperfusion (12, 17, 21). Thus the detrimental influence of tobacco smoking on cerebral I/R injury may be at least partially related to a decrease in MnSOD and UCP-2.

In summary, the present study found an increased superoxide level and a decrease in UCP-2 and MnSOD in the cerebral cortex and arteries of rats chronically exposed to nicotine. We speculate that mitochondrial oxidative stress may have important implications for the pathogenesis and pathophysiology of ischemic stroke. Novel therapeutic approaches reducing mitochondrial oxidative stress may be beneficial in the prevention and treatment of cardiovascular disease observed in smokers.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


