Superoxide dismutase restores endothelium-dependent arteriolar dilatation during acute infusion of nicotine

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Mayhan, William G., and Glenda M. Sharpe. Superoxide dismutase restores endothelium-dependent arteriolar dilatation during acute infusion of nicotine. J. Appl. Physiol. 85(4): 1292–1298, 1998.—We previously showed [Am. J. Physiol. 272 (Heart Circ. Physiol. 41): H2337–H2342, 1997] that nicotine impairs endothelium-dependent arteriolar dilatation. However, mechanisms that accounted for the effect of nicotine on endothelium-dependent vasodilatation were not examined. Thus the goal of this study was to examine the role of oxygen radicals in nicotine-induced impairment of arteriolar reactivity. We measured diameter of cheek pouch resistance arterioles (~50 µm diameter) in response to endothelium-dependent (ACh and ADP) and -independent (nitroglycerin) agonists before and after infusion of vehicle or nicotine in the absence or presence of superoxide dismutase. ACh, ADP, and nitroglycerin produced dose-related dilatation of cheek pouch arterioles before infusion of vehicle or nicotine. Infusion of vehicle, in the absence or presence of superoxide dismutase (150 U/ml), did not alter endothelium-dependent or -independent arteriolar dilatation. In contrast, infusion of nicotine (2 µg·kg−1·min−1) impaired endothelium-dependent, but not -independent, arteriolar dilatation. In addition, the effect of nicotine on endothelium-dependent vasodilatation was reversed by topical application of superoxide dismutase. We suggest that nicotine impairs endothelium-dependent arteriolar dilatation via an increase in the synthesis/release of oxygen-derived free radicals.

acetylcholine; adenosine 5′-diphosphate; nitroglycerin; cheek pouch; hamsters; smoking; endothelium-derived relaxing factor; oxygen radicals

It is clear that cigarette smoking and the use of smokeless tobacco products contribute to the pathogenesis of cardiovascular disease (38). Cigarette smoking and products of cigarette smoke produce diffuse vascular injury in many organ systems (13, 20, 28, 57) and impair nitric oxide synthase-dependent dilatation of large peripheral arteries (8, 19, 21, 23, 46) and resistance arterioles (53). The components of cigarette smoke and smokeless tobacco that account for vascular abnormalities are not certain. Investigators have shown that nicotine has toxic effects on endothelium (24, 29, 31), and thus nicotine may play a key role in impaired nitric oxide synthase-dependent vasoreactivity observed in users of tobacco products. In support of this concept, we recently showed that intravenous infusion of nicotine, which produces a plasma concentration of nicotine similar to that observed in smokers (3, 5, 49, 54), impairs nitric oxide synthase-dependent dilatation of resistance arterioles (37). The precise mechanism by which nicotine impaired nitric oxide synthase-dependent vasoreactivity, however, was not investigated in our previous study (37).

In addition to producing morphological abnormalities of the endothelium that may directly alter vascular reactivity, nicotine, the components of cigarette smoke, and smokeless tobacco products may produce their toxic effects via the production of oxygen radicals (40). A recent study (18) found that treatment of smokers with vitamin C, an antioxidant, improved impaired endothelium-dependent reactivity of large peripheral arteries. In addition, treatment of isolated rabbit aortas with an oxygen radical scavenger attenuated cigarette smoke-induced impairment of arterial relaxation (46). However, no studies have directly examined the role of oxygen radicals in nicotine-induced impairment of endothelium-dependent reactivity of resistance arterioles. An understanding of the effects of disease states on resistance blood vessels is critical, since these vessels regulate tissue perfusion. Thus the goal of the present study was to examine the role of oxygen radicals in nicotine-induced impairment of endothelium-dependent reactivity of resistance arterioles.

METHODS

Preparation of animals. Adult male hamsters (n = 20) weighing 120–140 g were anesthetized with pentobarbital sodium (6 mg/100 g body wt ip). A tracheotomy was performed to facilitate spontaneous breathing. A catheter was placed in a femoral artery for the measurement of arterial blood pressure. A catheter was placed in a femoral vein for injection of supplemental anesthesia (2–4 mg·100 g−1·h−1) and nicotine. At the end of the experiment, all animals were killed with an intravenous infusion of the anesthetic (60 mg/100 g body wt). All procedures were carried out after institutional approval and within institutional guidelines.

To visualize the microcirculation of the cheek pouch, we used a method that we described previously (36). Briefly, the left cheek pouch was spread gently over a small plastic baseplate and an incision was made in the skin to expose the cheek pouch membrane. An upper chamber was positioned over the baseplate and used to maintain the suffusion fluid. This arrangement forms a triple-layered complex: the baseplate, the upper chamber, and the cheek pouch membrane exposed between the two plates.

After these initial procedures, the hamster was transferred to a heated microscope stage. Body temperature was monitored and maintained constant via a feedback controller and heating pad. The cheek pouch chamber was connected to a reservoir that allows continuous suffusion of the cheek pouch microcirculation with bicarbonate buffer (36–38°C, pH 7.40). The chamber was also connected via a three-way valve to an
infusion pump that allowed for the controlled administration of vasoactive agonists.

Measurement of arteriolar diameter. The cheek pouch microcirculation was epi-illuminated with a fiber-optic light source and viewed through an Olympus microscope. The image of the cheek pouch microcirculation was projected through the microscope and into a closed-circuit television system that consists of television camera (model WV-1500, Panasonic), a monitor (model TR-124 MA, Panasonic), and a videotape recorder (model AG-1230, Panasonic). The diameter of cheek pouch arterioles was measured on-line using a video image shearing monitor (model 908, Instrumentation for Physiology and Medicine).

Agonists were mixed in the bicarbonate buffer and then superfused over the cheek pouch microcirculation. Application of agonists was randomized. Diameter of cheek pouch arterioles (1 arteriole/hamster) was measured immediately before application of agonists and every minute during a 5-min application period. Steady-state responses to agonists were reached within 2–3 min after the application was started, and the diameter of arterioles returned to baseline within 2 min after application of agonists was stopped. Application of agonists did not alter blood pressure. We used steady-state responses of arterioles to the agonists to compare the effects of nicotine on vascular reactivity. We examined responses of second-order arterioles to consecutive application of different doses of the same agonist, and application of different agonists was separated by 10–20 min. We chose to examine Reactivity of second-order arterioles, since they are important resistance vessels in the hamster cheek pouch (9, 10, 27), and thus changes in diameter of these vessels directly regulate tissue perfusion.

Experimental protocol. In all groups of hamsters the cheek pouch microcirculation was superfused for 30 min before responses of arterioles to the agonists were tested. In the first series of experiments, we examined the reproducibility of responses of arterioles to the agonists and the effect of nicotine on arteriolar reactivity. Thus, in the first group of hamsters (n = 6), we initially examined responses of cheek pouch arterioles to endothelium-dependent agonists (0.1 and 1.0 µM ACh and 1.0 and 10 µM ADP) and an endothelium-independent agonist (0.1 and 1.0 µM nitroglycerin). Then we started an intravenous infusion of vehicle (saline). Thirty minutes after starting infusion of vehicle, we again examined responses of arterioles to ACh, ADP, and nitroglycerin.

In a second group of hamsters (n = 5), we followed a protocol for the measurement of arteriolar reactivity similar to that described above, except we infused nicotine (2.0 µg·kg⁻¹·min⁻¹ iv for 30 min followed by a maintenance dose of 0.35 µg·kg⁻¹·min⁻¹) Thirty minutes after starting infusion of nicotine, we again examined responses of arterioles to ACh, ADP, and nitroglycerin.

In a second series of experiments, we examined the effect of superoxide dismutase on arteriolar reactivity during infusion of vehicle or nicotine. Thus, in a third group of hamsters (n = 5), we initially examined responses of cheek pouch arterioles to ACh, ADP, and nitroglycerin. Then we started a continuous topographical application of superoxide dismutase (150 U/ml) over the cheek pouch microcirculation. This concentration of superoxide dismutase has been shown to be efficacious (11, 45). Fifteen minutes after starting the application of superoxide dismutase, we started the intravenous infusion of vehicle. Thirty minutes after starting infusion of vehicle, we again examined responses of arterioles to ACh, ADP, and nitroglycerin.

In a fourth group of hamsters (n = 5), we followed a protocol for examining the effects of superoxide dismutase on arteriolar reactivity similar to that described above, except we infused nicotine in place of vehicle. Thus, in these studies, we initially examined responses of cheek pouch arterioles to the agonists. Then we started a continuous topical application of superoxide dismutase (150 U/ml) over the cheek pouch microcirculation. Fifteen minutes later, we started the intravenous infusion of nicotine (2.0 µg·kg⁻¹·min⁻¹ iv for 30 min followed by a maintenance dose of 0.35 µg·kg⁻¹·min⁻¹). Thirty minutes after starting infusion of nicotine, we again examined responses of arterioles to ACh, ADP, and nitroglycerin.

Drugs. ACh, ADP, nicotine (−)-1 methyl-2-[3-pyridyl]-pyrrolidine, and superoxide dismutase were purchased from Sigma Chemical (St. Louis, MO). Nitroglycerin was purchased from Sololak Laboratories (Elk Grove Village, IL). All stock solutions of agonists were prepared with saline.

Statistical analysis. Two-way ANOVA with repeated measures was used to compare responses of arterioles before and after intravenous infusion of vehicle or nicotine in the absence or presence of superoxide dismutase. Values are means ± SE. P ≤ 0.05 was considered to be significant.

RESULTS

Responses in the absence of superoxide dismutase. Baseline diameter of cheek pouch arterioles before suffusion of agonists and infusion of vehicle was 53 ± 2 µm. Topical application of ACh, ADP, and nitroglycerin produced dose-related dilatation of cheek pouch arterioles before the infusion of vehicle (Fig. 1). Infusion of vehicle did not alter baseline diameter of cheek pouch arterioles (51 ± 2 and 50 ± 3 µm before and during infusion of vehicle, respectively, P > 0.05). Responses of cheek pouch arterioles to the agonists were similar before and after infusion of vehicle (Fig. 1). Thus repeated application of agonists to cheek pouch arterioles elicits reproducible arteriolar dilatation.

Baseline diameter of cheek pouch arterioles before suffusion of agonists and infusion of nicotine was 54 ± 1 µm. Topical application of ACh, ADP, and nitroglycerin produced dose-related dilatation of cheek pouch arterioles before the infusion of nicotine (Fig. 2). Intravenous infusion of nicotine did not alter baseline diameter of cheek pouch arterioles (53 ± 2 and 54 ± 1 µm before and during infusion of nicotine, respectively, P > 0.05). In contrast to that observed during infusion of vehicle, infusion of nicotine markedly impaired dilatation of cheek pouch arterioles to ACh and ADP (Fig. 2). However, infusion of nicotine did not alter responses of cheek pouch arterioles to nitroglycerin (Fig. 2). Thus infusion of nicotine produces selective impairment in endothelium-dependent, but not -independent, arteriolar dilatation. This is similar to our previous results (37).

Responses in the presence of superoxide dismutase. Baseline diameter of cheek pouch arterioles before suffusion of agonists, application of superoxide dismutase, and infusion of vehicle was 53 ± 3 µm. Topical
Application of ACh, ADP, and nitroglycerin produced dose-related dilatation of cheek pouch arterioles before the application of superoxide dismutase and infusion of vehicle (Fig. 3). Topical application of superoxide dismutase and infusion of vehicle did not alter baseline diameter of cheek pouch arterioles (50 ± 1 vs. 48 ± 1 µm, P > 0.05). In addition, responses of cheek pouch arterioles to the agonists were similar in the absence and presence of superoxide dismutase (Fig. 3). Thus it does not appear that superoxide dismutase alters reactivity of cheek pouch arterioles to ACh, ADP, and nitroglycerin during infusion of vehicle.

Baseline diameter of cheek pouch arterioles before suffusion of agonists, application of superoxide dismutase, and infusion of nicotine was 51 ± 2 µm. Topical application of ACh, ADP, and nitroglycerin produced dose-related dilatation of cheek pouch arterioles before the application of superoxide dismutase and infusion of nicotine (Fig. 4). Topical application of superoxide dismutase and infusion of nicotine did not alter baseline diameter of cheek pouch arterioles (48 ± 2 vs. 50 ± 2 µm, P > 0.05). In contrast to that observed during infusion of nicotine in the absence of superoxide dismutase (Fig. 2), topical application of superoxide dismutase prevented nicotine-induced impairment of endothelium-dependent arteriolar reactivity in response to ACh and ADP (Fig. 4). Superoxide dismutase did not alter responses of cheek pouch arterioles to nitroglycerin (Fig. 4). Thus it appears that superoxide dismutase can prevent impairment of endothelium-dependent arteriolar reactivity during infusion of nicotine.

DISCUSSION

The present study is the first to examine the role of oxygen radicals in nicotine-induced impairment of endothelium-dependent reactivity of resistance arterioles in vivo. We found that infusion of nicotine, at a concentration that we (37) previously showed to produce a plasma concentration of nicotine similar to that observed in smokers (4, 26, 41, 50, 54), markedly impaired endothelium-dependent dilatation of resistance arterioles. This finding could not be explained by a nonspecific effect of nicotine on vascular reactivity, since responses to nitroglycerin were not altered by infusion of nicotine. In addition, we found that treat-
...ment of the cheek pouch microcirculation with superoxide dismutase prevented nicotine-induced impairment of endothelium-dependent vasodilatation. We suggest that alterations in endothelium-dependent responses of blood vessels in smokers and users of smokeless tobacco products may be related to the production of oxygen-derived free radicals.

Consideration of methods. We examined reactivity of cheek pouch arterioles to ACh and ADP, which appear to stimulate the synthesis/release of nitric oxide or a nitric oxide-containing compound in a variety of vascular preparations (14). We also examined responses of cheek pouch arterioles to nitroglycerin, which produces vasodilatation independent of nitric oxide synthase (14). Previous studies have shown that arterioles of the hamster cheek pouch respond to and release nitric oxide or a nitric oxide-containing compound during application of endothelium-dependent mediators such as bradykinin and methacholine (51, 52). In addition, we have shown that dilatation of cheek pouch arterioles in response to ACh, but not nitroglycerin, can be attenuated by application of enzymatic inhibitors of nitric oxide synthase (35). Furthermore, changes in venular permeability in response to ADP can be inhibited by enzymatic inhibitors of nitric oxide synthase (34). Thus it appears that blood vessels of the hamster cheek pouch exhibit typical endothelium-dependent responses. We suggest that the synthesis/release of nitric oxide accounts for dilatation of cheek pouch arterioles in response to ACh and ADP.

To examine a potential role for oxygen radicals in impaired endothelium-dependent reactivity, we applied superoxide dismutase to the cheek pouch microcirculation. We found that application of superoxide dismutase restored endothelium-dependent, but did not alter endothelium-independent, vasodilatation after exposure to nicotine. A previous study (39) reports that inhibition of superoxide dismutase activity using diethyldithiocarbamate (DETC) reduced endothelium-dependent, vasodilatation after exposure to nicotine. A previous study (39) reports that inhibition of superoxide dismutase activity using diethyldithiocarbamate (DETC) reduced endothelium-dependent and -independent relaxation of the rabbit aorta. Thus it appeared that an increase in oxygen radical formation by DETC nonselectively impaired vascular relaxation. If nicotine increased the formation of oxygen radicals in the present study, then one might have predicted, on the basis of this previous study (39), that vasodilatation in response to nitroglycerin would have...
been altered before application of superoxide dismutase. However, we did not find an effect of nicotine or superoxide dismutase on nitroglycerin-induced vasoconstriction. The discrepancy between findings of the present study and that reported previously (39) regarding the effect of oxygen radicals on reactivity of blood vessels to a nitrovasodilator is not clear but may be related to species, size, and/or location of vessel examined and/or specificity of DETC on superoxide dismutase activity. The findings of the present study, however, are supported by many previous studies that have examined a role for oxygen radicals in impaired responses of blood vessels during various disease states. These previous studies have shown that treatment with scavengers of oxygen radicals restores endothelium-dependent, but does not alter endothelium-independent, reactivity (12, 43, 47, 48). In addition, responses of vessels to nitrovasodilators before treatment with oxygen radical scavengers were similar in control and diseased states (12, 43, 47, 48). Thus, in addition to the present study, these previous studies do not appear to support a role for oxygen radicals in modulating responses to nitrovasodilators. Because nitrovasodilators appear to produce relaxation of vascular smooth muscle via an intracellular enzymatic conversion to nitric oxide (1, 17, 25), we suggest that oxygen radicals formed by the infusion of nicotine may act extracellularly to inhibit the action of nitric oxide after the application of endothelium-dependent agonists.

We examined the acute effects of nicotine infusion on arteriolar reactivity. However, we believe that our model has important implications for the chronic effects of smoking on vascular reactivity. It is probable that nicotine concentration in habitual smokers is elevated throughout the course of the day. In support of this, a previous study has shown that the plasma level of nicotine is $25 \pm 6 \text{ ng/ml}$ at 6.5 h after ad libitum smoking (26). Furthermore, several previous studies have shown that plasma levels of nicotine are elevated in chronic smokers (4, 41, 50, 54). We showed previously (37) that infusion of nicotine at the concentration used in the present study produces a plasma level of nicotine similar to that observed in smokers (4, 26, 41, 50, 54). Thus, although we are examining the acute effects of nicotine infusion on arteriolar reactivity, we believe our studies may have implications for the chronic effects of cigarette smoking and the use of smokeless tobacco products on vascular reactivity.

Consideration of previous studies. Many previous studies have examined the effects of cigarette smoking and/or cigarette smoke extract on endothelium-dependent responses of large and small blood vessels. Studies using human subjects have shown that cigarette smoking (acute and chronic) impairs nitric oxide synthase-mediated relaxation of large blood vessels (18, 21–23, 56, 59). In addition, in a recent study, Ota et al. (46) found that cigarette smoke extract impaired endothelium-dependent relaxation of the rabbit aorta. Furthermore, we previously reported that cigarette smoke extract impairs endothelium-dependent, but not -independent, dilatation of cheek pouch arterioles (53). Finally, Suzuki et al. (58) reported that topical application of smokeless tobacco extract to the cheek pouch microcirculation impairs endothelium-dependent vasoconstriction. Thus it appears that cigarette smoking, the components of cigarette smoke, and smokeless tobacco impairs nitric oxide synthase-dependent dilatation of large and small blood vessels.

Few studies have examined the direct effects of nicotine on reactivity of blood vessels. Martz et al. (33) found that intraperitoneal injection of nicotine produced dilatation of arterioles contained within the wing microcirculation. In contrast, Schilling et al. (55) found that topical application of nicotine did not alter baseline diameter of cerebral arterioles in cats. In addition, Myers et al. (42) found that nicotine (acute or chronic application) did not alter baseline diameter or constriction of cheek pouch arterioles in response to norepinephrine. Furthermore, we found that intravenous infusion of nicotine in hamsters did not alter baseline diameter of cheek pouch arterioles, heart rate, or blood pressure (37). However, infusion of nicotine markedly inhibited endothelium-dependent, but not -independent, dilatation of cheek pouch arterioles (37). Thus findings of the present study are in agreement with previous studies (37, 42, 55), in that we did not find an effect of nicotine on baseline diameter of arterioles. The findings of the present study extend that of previous studies by examining mechanisms by which nicotine alters endothelium-dependent reactivity of resistance arterioles.

Mechanisms that may contribute to altered vascular reactivity during smoking and/or exposure to smoke products are complex. First, cigarette smoke and/or the components of cigarette smoke (including nicotine) have been shown to produce morphological abnormalities of the endothelium, which may directly affect endothelial function, i.e., synthesis/release of vasoactive substances such as nitric oxide (2, 6, 7, 32). Second, many toxic compounds that may affect endothelial function are contained in cigarette smoke. Acrolein and acetaldehyde are highly soluble components of cigarette smoke extract and have been shown to cause vascular damage (15, 16, 30, 44). However, no studies have examined the effects of acrolein and acetaldehyde on endothelium-dependent vascular reactivity. In addition to acrolein and acetaldehyde, cigarette smoke and cigarette smoke extract appear to contain oxygen radicals (40, 46). Increased formation of oxygen radicals could directly damage the endothelium, affecting the synthesis/release of nitric oxide, and/or scavenge nitric oxide to affect vascular reactivity. In support of this concept, a recent study (18) found that treatment of chronic smokers with vitamin C, an antioxidant, could improve impaired endothelium-dependent reactivity of large arteries. In addition, Ota et al. (46) reported that treatment with a scavenger of oxygen radicals attenuated cigarette smoke extract-induced impairment of endothelium-dependent relaxation of the rabbit aorta. Thus it appears that the formation of oxygen radicals may play a critical role in impaired endothelium-
dependent responses of large blood vessels during exposure to smoke products. Third, a recent study showed that impaired reactivity of large peripheral arteries in smokers is related to an alteration in the activity of endothelial nitric oxide synthase (22). Fourth, we have suggested that impaired reactivity of cheek pouch resistance arteries to endothelium-dependent agonists after exposure to cigarette smoke extract could be restored toward normal by treatment with indo- methacin (53). Thus impaired endothelium-dependent vasodilatation in response to application of cigarette smoke extract appeared to be related to the synthesis/release of substances (including oxygen radicals) produced via the cyclooxygenase pathway.

Although we suggest that the formation of oxygen radicals is important in nicotine-induced impairment of endothelium-dependent responses of arterioles, the precise source of oxygen radicals cannot be determined by the present study. However, in light of studies which suggest that nicotine produces endothelial damage (31), it is possible that the damaged endothelium may be a source of oxygen radicals. Because indomethacin could restore cigarette smoke extract-induced impairment of endothelial dysfunction (53), it is possible that nicotine could activate the cyclooxygenase pathway in endothelium and induce the formation of oxygen radicals. Furthermore, because nicotine may accelerate the development of atherosclerosis, it is possible that stimulation of various vascular elements (e.g., platelets and macrophages) by nicotine may produce an increase in oxygen radical formation. Regardless of the source of oxygen radicals, the findings of the present study appear to agree with previous studies (18, 46), which have suggested an important role for oxygen radicals in impaired reactivity of large peripheral blood vessels during smoking and/or exposure to products of cigarette smoke. Furthermore, the findings of the present study extend that of previous studies (18, 46) by examining the component contained in smoke products that alters endothelium-dependent vascular reactivity and by examining responses of resistance blood vessels, which directly regulate tissue perfusion.

In summary, we examined the role of oxygen radicals in nicotine-induced impairment of nitric oxide synthase-dependent reactivity of resistance arterioles. We found that infusion of nicotine impaired reactivity of arterioles to ACh and ADP but not to nitroglycerin. In addition, treatment with superoxide dismutase prevented nicotine-induced impairment of nitric oxide synthase-dependent arteriolar reactivity. We suggest that the formation of oxygen radicals contributes to impairment of endothelium-dependent vasoreactivity, and perhaps cardiovascular disease, observed in users of tobacco products.

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