Nicotine impairs histamine-induced increases in macromolecular efflux: role of oxygen radicals

William G. Mayhan and Glenda M. Sharpe. Nicotine impairs histamine-induced increases in macromolecular efflux: role of oxygen radicals. J. Appl. Physiol. 84(5): 1589–1595, 1998.—Nicotine, a major component of cigarettes and smokeless tobacco, has toxic effects on endothelium and impairs reactivity of resistance arterioles in response to agonists that stimulate the synthesis and/or release of nitric oxide. However, the effect of nicotine on nitric oxide synthase-dependent increases in macromolecular transport is not known. Thus our first goal was to determine the effect of nicotine on histamine-induced increases in macromolecular efflux. We used intravital microscopy and FITC dextran (mol wt 70,000) (FITC-dextran-70K) to examine macromolecular extravasation from postcapillary venules in response to histamine before and after intravenous infusion of vehicle or nicotine. Extravasation of macromolecules was quantitated by counting venular leaky sites and calculating clearance (ml/s × 10^{-6}) of FITC-dextran-70K. Histamine elicited reproducible increases in venular leaky sites and clearance in hamsters infused with vehicle. In contrast, nicotine infusion inhibited histamine-induced increases in macromolecular efflux. Histamine (1.0 and 5.0 µM) elicited 19 ± 2 and 34 ± 4 vs. 3 ± 1 and 11 ± 5 leaky sites per 0.11 cm^2, before vs. after nicotine infusion, respectively (P < 0.05). Histamine-induced clearance of FITC-dextran-70K was also impaired after infusion of nicotine. Our second goal was to examine whether alterations in histamine-induced increases in macromolecular efflux by nicotine may be related to the production of oxygen radicals. Application of superoxide dismutase (150 U/ml) to the hamster cheek pouch restored histamine-induced increases in venular leaky sites and clearance of FITC-dextran-70K during infusion of nicotine. Thus nicotine alters agonist-induced increases in microvascular permeability, via the formation of oxygen radicals, to presumably inactivate nitric oxide.

CIGARETTE SMOKING in human subjects produces morphological and functional alterations in blood vessels. Morphological abnormalities, i.e., endothelial cell swelling, extensive subendothelial edema, and subendothelial blebs, and an increased number of subendothelial macrophages in the arterial wall have been observed in animals and human subjects exposed to cigarette smoke (3, 7, 8, 21). Functionally, studies have shown that exposure of animals and endothelial cell monolayers to cigarette smoke increases basal permeability to plasma proteins (4, 16, 18, 21) and alters nitric oxide synthase-mediated relaxation of large peripheral arteries (13, 14) and dilatation of resistance arterioles (36).

One of the major components of cigarette smoke is nicotine. Nicotine is a toxic compound and has been shown to produce diffuse damage to endothelium (7, 15, 20). In addition, our laboratory (26) has recently shown that infusion of nicotine in hamsters, at levels similar to those observed in chronic smokers (5, 6, 34, 37), impairs nitric oxide synthase-mediated dilatation of resistance arterioles. Thus it appears that circulating levels of nicotine contribute to altered endothelial function of the microcirculation during cigarette smoking.

Many inflammatory mediators, including histamine, produce an increase in permeability of the microcirculation specifically at the level of postcapillary venules (19, 25, 35). We (22–24) and others (17, 41, 42) have shown that increases in venular permeability in response to inflammatory mediators, including histamine, are related to the synthesis and/or release of nitric oxide or a nitric oxide-containing compound. Because it appears that nicotine may alter nitric oxide synthase-mediated responses of resistance arterioles (26), we reasoned that nicotine may alter nitric oxide synthase-mediated increases in macromolecular permeability. Thus the first goal of this study was to examine the effect of intravenous infusion of nicotine on histamine-induced increases in macromolecular efflux from venules of the hamster cheek pouch.

Recently, investigators have shown that the toxic effects of cigarette smoking may be related to the production of oxygen-derived free radicals (12, 29, 33). Thus the second goal of our study was to examine whether nicotine-induced impairment in nitric oxide synthase-mediated increases in venular permeability may be related to the synthesis and/or release of oxygen-derived free radicals to presumably inactivate nitric oxide.

MATERIALS AND METHODS

Preparation of animals. Adult male hamsters weighing 120–160 g were anesthetized with pentobarbital sodium (6 mg/100 g body wt ip), and a tracheotomy was performed to facilitate spontaneous breathing. The left femoral vein was cannulated for the purpose of injecting the intravascular tracer [FITC dextran (mol wt 70,000) (FITC-dextran-70K)] and supplemental anesthesia (2–4 mg: 100 g^{-1} h^{-1}). The left femoral artery was cannulated for the purpose of measuring arterial pressure, which remained constant during the experimental period.

To visualize the microcirculation of the cheek pouch, we used a method that our laboratory (25) described previously. Briefly, the cheek pouch was spread over a small plastic baseplate, and an incision was made in the skin to expose the cheek-pouch membrane. An avascular layer of connective tissue was excised to expose the microvessels of the cheek pouch. An upper chamber was then positioned over the baseplate to provide a reservoir for the suffusion fluid. This arrangement forms a triple-layered complex: the baseplate, the upper chamber, and the cheek-pouch microcirculation exposed between these two plates.
The cheek-pouch microcirculation was continuously superfused with a bicarbonated buffer (37 ± 1°C). The cheek-pouch chamber also was connected via a three-way valve to a pump that allowed for infusion of agonists (histamine and/or superoxide dismutase) into the suffusate. This method, which our laboratory (25) has used previously, allowed us to maintain the suffusion fluid at a constant temperature and pH during application of agonists.

Evaluation of microvascular permeability. Alterations in macromolecular efflux were quantitated by counting the number of microvascular leaky sites, which occurred exclusively around postcapillary venules (2). The number of leaky sites (per 0.11 cm²) was determined under control conditions (before application of agonists) and at 1, 3, 5, 7, 10, 15, 20, and 30 min during and after a 5-min application of histamine (1.0 and 5.0 µM). The maximum number of leaky sites observed in two microscopic fields (0.11 cm² per microscopic field) under control conditions and during application of agonists was averaged, and we report this average value (22).

In some hamsters, we examined the clearance of FITC-dextran-70K in response to histamine (1.0 and 5.0 µM). Thus, in these experiments, the suffusion fluid was collected in glass test tubes at 5-min intervals with the aid of a fraction collector (Micro-fractionator, Gilson Medical Electronics, Middleton, WI). In addition, arterial blood samples were collected in heparinized capillary tubes (50–70 µl vol) before and at various intervals after injection of FITC-dextran-70K.

To quantify the concentration of FITC-dextran-70K in the plasma and suffusate, we performed a standard curve for FITC-dextran-70K concentration vs. percent emission on a spectrophotofluorometer (model LS-30; Perkin-Elmer, Norwalk, CT). The standard was FITC-dextran-70K, which was prepared on a weight-per-volume basis. With the use of bicarbonated buffer as background, a standard curve was generated for each experiment, and each curve was subjected to linear-regression analysis. The percent emission for unknown samples (plasma and suffusate) was measured on the spectrophotofluorometer, and the concentration of FITC-dextran-70K was calculated from the standard curve. Clearance of FITC-dextran-70K was determined by calculating the ratio of suffusate (ng/ml) to plasma (ng/ml) concentration of FITC-dextran-70K and multiplying this ratio by the suffusate flow rate (2 ml/min) (25).

Measurement of plasma nicotine. In some vehicle- (n = 3) and nicotine-treated (n = 6) hamsters, plasma samples (3 ml) were drawn at the end of the experimental protocol. The concentration of nicotine and its major metabolite, cotinine, in plasma was determined by using gas chromatography (MedTox Laboratories; St. Paul, MN).

Experimental protocol. In the first series of experiments, we examined the reproducibility of changes in macromolecular efflux in response to histamine. Thus, after insertion of the cheek-pouch chamber, the preparation was allowed to equilibrate for 40–45 min. Then successive doses of histamine (1.0 and 5.0 µM (n = 9 and 6 animals tested, respectively)) were superfused over the cheek-pouch microcirculation for 5 min. The number of venular leaky sites and clearance of FITC-dextran-70K were determined as described in Evaluation of microvascular permeability. After examining the effects of histamine on macromolecular transport, we then started a continuous 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 for the duration of the experiment). Our laboratory (26) has shown previously that this procedure produces plasma levels of nicotine similar to those observed in smokers (5, 6, 34, 37). Thirty minutes after starting infusion of nicotine, we again examined the effects of histamine on macromolecular efflux.

In a third series of experiments (n = 6 animals tested), we examined the role of oxygen radicals in nicotine-induced impairment in microvascular efflux in response to histamine. After insertion of the cheek-pouch chamber, the preparation was allowed to equilibrate for 40–45 min. Then histamine (5.0 µM) was superfused over the cheek-pouch microcirculation for 5 min. The number of venular leaky sites and clearance of FITC-dextran-70K were determined as described in Evaluation of microvascular permeability. After examining the effect of histamine on macromolecular transport, we then started a continuous superfusion of superoxide dismutase (150 U/ml) over the cheek-pouch microcirculation. This concentration of superoxide dismutase has been shown to be efficacious (10, 32). Ten to fifteen minutes after starting the superfusion of superoxide dismutase, 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 for the duration of the experiment). Thirty minutes later, we again examined the effects of histamine on macromolecular efflux. Application of superoxide dismutase (150 U/ml) does not alter histamine-induced increases in venular macromolecular efflux in the absence of nicotine (Mayhan and Sharpe, unpublished observation).

Statistical analysis. A paired t-test was used to compare the number of venular leaky sites and clearance of FITC-dextran-70K in response to repeated application of histamine with or without treatment with nicotine. A paired t-test was used to compare the number of venular leaky sites and clearance of FITC-dextran-70K before and after treatment with superoxide dismutase in the presence of nicotine. A P value of 0.05 was considered to be significant.

RESULTS

Responses in vehicle-treated hamsters. In vehicle-treated hamsters, there were no leaky sites visible, and clearance of FITC-dextran-70K was minimal (0.20 ± 0.05 ml/s × 10⁻⁶) before application of histamine. Infusion of vehicle did not alter the formation of venular leaky sites in response to histamine (Fig. 1). Thus topical application of histamine (1.0 and 5.0 µM) produced a reproducible dose-related increase in the number of venular leaky sites. Leaky sites occurred exclusively around postcapillary venules, reached a maximum within 5–7 min after starting suffusion of histamine, and resolved during the recovery period. Application of histamine (1.0 and 5.0 µM) also produced a reproducible increase in the clearance of FITC-dextran-70K from the cheek-pouch microcirculation.
The increase in clearance of FITC-dextran-70K in response to histamine also resolved during the recovery period. Thus there appears to be a relationship between venular leaky sites and transport of macromolecules across postcapillary venules under control conditions and during stimulation with histamine. These findings are similar to those reported previously by our laboratory (25).

Responses in nicotine-treated hamsters. Plasma concentrations of nicotine and cotinine in nicotine-treated hamsters were 24 ± 4 and 84 ± 13 ng/ml, respectively. In contrast, plasma nicotine and cotinine levels were negligible (<1 ng/ml) in hamsters infused with vehicle (saline).

There were no leaky sites visible before treatment with nicotine, and the clearance of FITC-dextran-70K was minimal (0.22 ± 0.05 ml/s × 10^-6) before application of histamine. Before intravenous infusion of nicotine, application of histamine produced a dose-related increase in venular leaky sites (Fig. 3) and clearance of FITC-dextran-70K (Fig. 4). These findings were similar in magnitude to those observed in vehicle-treated hamsters (Figs. 1 and 2; P > 0.05). Before application of histamine, intravenous infusion of nicotine did not produce venular leaky sites (0 ± 0) and did not increase the clearance of FITC-dextran-70K (0.28 ± 0.08 ml/s × 10^-6). However, intravenous infusion of nicotine significantly decreased the formation of venular leaky sites (Fig. 3) and clearance of FITC-dextran-70K (Fig. 4) in response to application of histamine. Thus it appears that histamine-induced, nitric oxide synthase-dependent increases in macromolecular efflux are impaired during infusion of nicotine.
Effect of superoxide dismutase. Before infusion of nicotine and superfusion with superoxide dismutase (150 U/ml), there were no leaky sites visible (0 ± 0), and the clearance of FITC-dextran-70K was minimal (0.32 ± 0.11 ml/s × 10^-2). Before infusion of nicotine and superfusion with superoxide dismutase, topical application of histamine (5.0 µM) produced an increase in the formation of venular leaky sites and clearance of FITC-dextran-70K (Fig. 5). Superfusion with superoxide dismutase and infusion of nicotine did not produce an increase in venular leaky sites or clearance of FITC-dextran-70K. In contrast to the results observed with intravenous infusion of nicotine alone (Figs. 3 and 4), in the presence of superoxide dismutase, nicotine infusion no longer impaired histamine-induced formation of venular leaky sites and clearance of FITC-dextran-70K (Fig. 5). Thus it appears that oxygen radicals may play an important role in nicotine-induced impairment of macromolecular efflux in response to histamine.

DISCUSSION

The results of the present study suggest that nicotine impairs histamine-induced increases in venular permeability, which are presumably related to an increased synthesis and/or release of nitric oxide or a nitric oxide-containing compound (24, 42). In addition, our findings suggest that the mechanism of impaired agonist-induced increases in macromolecular extravasation during infusion of nicotine appears to be related to the synthesis and/or release of oxygen radicals to presumably inactive nitric oxide.

Consideration of methods. The hamster cheek-pouch microcirculation has been used by many investigators to examine the effects of inflammatory mediators on macromolecular transport (25, 28, 38, 39). In the present study, we quantitated changes in microvascular efflux using two methods. First, we counted the number of venular leaky sites before and during superfusion with histamine in the absence and presence of nicotine. These leaky sites represent areas of fluorescent dextran extravasation and occur exclusively around post-capillary venules. Second, we calculated the clearance of FITC-dextran-70K to verify that changes in leaky-site formation during application of histamine produce an increase in the transport of macromolecules across the venular membrane. Our laboratory (25) has shown previously that there is a relationship between the number of venular leaky sites formed in response to an inflammatory mediator and the clearance of fluorescent tracer across the venular endothelium. Thus these methods allowed us to quantitate the location and magnitude of changes in macromolecular efflux during stimulation with histamine.

We considered the possibility that alterations in histamine-induced increases in venular leaky sites and clearance of FITC-dextran-70K in hamsters treated with nicotine may be related to the effects of nicotine on arteriolar diameter and, thus, venular driving pressure. Recently, our laboratory (26) has shown that intravenous infusion of nicotine impaired nitric oxide synthase-mediated dilatation of cheek-pouch arterioles in response to acetylcholine and adenosine 5'-diphosphate. Thus it is conceivable that, if nicotine also altered histamine-induced arteriolar dilatation, which appears to be related to the synthesis and/or release of nitric oxide (27), then venular driving pressure may be decreased. However, previous studies have suggested that changes in vascular diameter and/or venular driving pressure do not account for venular leaky-site formation following application of inflammatory mediators (23, 30, 40). In addition, other investigators have shown that inflammatory mediators increase the permeability of isolated venules, in which driving pressure is presumably constant (11, 42). Thus we suggest that inhibition of the formation of venular leaky sites and clearance of FITC-dextran-70K in response to histamine in hamsters treated with nicotine are probably

![Figure 5. Number of venular leaky sites (per 0.11 cm²; left) and change in clearance of FITC-dextran-70K (ml/s × 10^-2; right) from baseline in response to application of histamine (5.0 µM, n = 6 hamsters) before (in absence of superoxide dismutase [SOD; 150 U/ml]; open bars) and during (hatched bars) infusion of nicotine in presence of SOD. Values are means ± SE.](http://jap.physiology.org/)
not related to the effects of nicotine on arteriolar dilatation and/or venular driving pressure.

We examined the possibility that oxygen radicals accounted for nicotine-induced impairment of macromolecular efflux in response to histamine. We thought that, if nicotine increased the synthesis and/or release of oxygen radicals, then we might observe an increase in basal macromolecular efflux during infusion of nicotine. Others have shown that systems which increase the production of oxygen radicals (xanthine plus xanthine oxidase) increase the permeability of the hamster cheek-pouch microcirculation (9). However, we found that infusion of nicotine did not produce an increase in venular leaky sites or increase the clearance of FITC-dextran-70K. At least two possibilities may explain this finding. First, the duration of exposure to nicotine may not have been sufficient enough to produce a change in the integrity of the cheek-pouch membrane to a large-molecular-weight tracer such as FITC-dextran-70K. Thus longer periods of exposure to nicotine may be required to alter the transport characteristics of the cheek-pouch membrane. Second, the concentration of oxygen radicals needed to increase basal permeability may be different than that required to inactivate nitric oxide. Although it is not possible for us to determine precisely which of these factors accounts for our results, we suggest that our findings are important in that they implicate a role for oxygen radicals in impaired agonist-induced increases in venular permeability during infusion of nicotine.

Consideration of previous studies. Few studies have examined the effect of nicotine on vascular permeability. A study by Lin et al. (20) examined the long-term effect of nicotine on endothelial cell death and macromolecular transport. These investigators reported that administration of nicotine to rats (given via the drinking water) for 6 wk enhances macromolecular transport of albumin across the thoracic aorta. In contrast, a study by Allen et al. (1) found that intravenous infusion of nicotine in dogs did not alter the transport of fibrinogen across the femoral artery. Similarly, a study by Myers et al. (31) found that intravenous infusion of nicotine did not alter the basal transport of FITC-dextran across the hamster cheek-pouch microcirculation. The findings of the present study support those of other investigators (1, 31). We found that intravenous infusion of nicotine did not alter basal macromolecular efflux across the cheek-pouch microcirculation. The discrepancy between the present study and the previous study (20), which suggests that nicotine may alter basal permeability, may be related to duration of nicotine exposure and/or plasma levels of nicotine obtained. In the previous study (20), nicotine was given via the drinking water for a period of 6 wk. However, in the present study, we examined the acute effects of nicotine on macromolecular efflux. In addition, in the previous study (20), plasma levels of nicotine increased to >1,000 ng/ml, which is far greater than those reported in chronic cigarette smokers (10–40 ng/ml) (5, 6, 34, 37) and the ones reported in the present study. Thus it is possible that very high concentrations of nicotine given over long periods of time would produce changes in the integrity of the endothelial barrier.

One previous study has examined the effect of nicotine on histamine-induced changes in permeability of the hamster cheek-pouch microcirculation (31). These investigators reported that intravenous infusion of nicotine significantly potentiated the formation of venular leaky sites and clearance of FITC-dextran (31). The findings of the present study differ from those of this previous study (31). We report that intravenous infusion of nicotine decreases the formation of venular leaky sites and clearance of FITC-dextran-70K. The discrepancy between the present and the previous study (31) may again relate to plasma levels of nicotine. In the previous study (31), plasma nicotine was 182 ng/ml. This value is many times greater than that observed in chronic smokers (5, 6, 34, 37) and in the present study. Thus we suggest that nicotine may have dose-related effects on the vasculature. It is possible that extreme levels of nicotine may alter various cellular pathways, which may account for differences observed in studies that have examined pharmacological levels vs. levels of nicotine observed in chronic smokers.

Mechanism for inhibitory effect of nicotine. Although no previous studies have examined the role of oxygen radicals in nicotine-induced impairment of nitric oxide synthase-mediated increases in venular permeability, previous studies have examined the effects of cigarette smoking and cigarette-smoke extract on nitric oxide synthase-mediated vasoreactivity (12, 29, 33). A study by Murohara et al. (29) reported that contraction of porcine coronary arteries in response to cigarette-smoke extract can be attenuated by inhibition of oxygen radical production. A study by Heitzer et al. (12) reported that the antioxidant vitamin C improves impaired acetylcholine-induced dilatation in chronic smokers. Finally, a study by Ota et al. (33) reported that impaired nitric oxide synthase-mediated relaxation of the aorta in rabbits after treatment with cigarette-smoke extract can be attenuated by treatment with oxygen radical scavengers. Thus it appears that the components of cigarette smoke attenuate nitric oxide synthase-mediated vasoreactivity via the synthesis and/or release of oxygen radicals, to presumably inactivate nitric oxide. The findings of the present study agree with those of these previous studies (12, 29, 33). We found that treatment of the hamster cheek-pouch microcirculation with superoxide dismutase restored nitric oxide synthase-mediated, histamine-induced increases in venular permeability in animals in which we infused nicotine. Thus it appears that the production of oxygen radicals is important in impaired agonist-induced increases in venular permeability during infusion with nicotine. While we suggest that oxygen radicals contribute to nicotine-induced impairment of macromolecular efflux, the source of oxygen radicals cannot be determined from our studies.

In conclusion, this is the first study to examine the effect of nicotine, at levels seen in chronic smokers, on nitric oxide synthase-dependent increases in venular permeability. We found that infusion of nicotine im-
pairs histamine-induced increases in venular macromolecular efflux. In addition, impaired histamine-induced increases in venular permeability during infusion of nicotine could be restored by treatment with superoxide dismutase. Thus it appears that the synthesis and/or release of oxygen radicals, to presumably inactivate nitric oxide, contributes to nicotine-induced impairment of macromolecular efflux.

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