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TIME-DEPENDENT INHIBITION AND TETRAHYDROBIOPTERIN DEPLETION OF ENDOTHELIAL NITRIC-OXIDE SYNTHASE CAUSED BY CIGARETTES

Ezra R. Lowe, Andrew C. Everett, Anthony J. Lee, Miranda Lau, Anwar Y. Dunbar, Vladimir Berka, Ah-lim Tsai, and Yoichi Osawa

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan (E.R.L., A.C.E., A.J.L., M.L., A.Y.D., Y.O.); and Department of Internal Medicine, University of Texas, Health Sciences Center, Houston, Texas (V.B., A.-L.T.)

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ABSTRACT:

Smoking causes a dysfunction in endothelial nitric-oxide synthase (eNOS), which is ameliorated, in part, by administration of tetrahydrobiopterin (BH_4). The exact mechanism by which the nitric oxide deficit occurs is unknown. We have previously shown that aqueous extracts of chemicals in cigarettes (CE) cause the suicide inactivation of neuronal NO synthase (nNOS) by interacting at the substrate-binding site. In the current study, we have found that CE directly inactivates eNOS by a process that is not affected by the natural substrate L-arginine and is distinct from the mechanism of inactivation of nNOS. We discovered that CE causes a time-, concentration-, and NADPH-dependent inactivation of eNOS in an in vitro system containing the purified enzyme, indicating a metabolic component to the inactivation. The CE-treated eNOS but not nNOS was nearly fully reactivated upon incubation with excess BH_4, suggesting that BH_4 depletion is a potential mechanism of inactivation. Moreover, in the presence of CE, eNOS catalyzed the oxidation of BH_4 to dihydrobiopterin and biopterin by a process attenuated by high concentrations of superoxide dismutase but not catalase. We speculate that a redox active component in CE, perhaps a quinone compound, causes oxidative uncoupling of eNOS to form superoxide, which in turn oxidizes BH_4. The discovery of a direct inactivation of eNOS by a compound(s) present in tobacco provides a basis not only for further study of the mechanisms responsible for the biological effects of tobacco but also a search for a potentially novel inactivator of eNOS.

Cigarette smoking is dose dependently associated with impairment of endothelial-dependent dilation in humans (Adams et al., 1997). The role of NO deficit in the genesis of vascular disease has been reviewed (Cooke and Dzau, 1997b); moreover, it is known that acute as well as chronic smoking decreases exhaled NO in humans (Kharitonov et al., 1995). A variety of mechanisms have been postulated to explain this NO deficit (Cooke and Dzau, 1997a), including the enhanced formation of superoxide, which would rapidly react with NO and thereby decrease its bioactivity (Kelm et al., 1997). More recently, Heitzer et al. (2000) reported that administration of tetrahydrobiopterin (BH_4) to chronic smokers improves endothelium-dependent vasodilation as determined by forearm blood-flow measurements. Administration of tetrahydroneopterin, which has the same antioxidant properties as BH_4 but is not a cofactor for NO synthase, did not improve vascular function and further supports the notion of a specific dysfunction in NOS rather than a general enhancement of oxidative stress (Heitzer et al., 2000). In addition, the vasodilator response due to sodium nitroprusside is not affected in these smokers irrespective of BH_4 administration, indicating that the responsiveness to NO is not changed. Thus, taken together, these observations strongly implicate a dysfunctional NO synthase in smokers (Heitzer et al., 2000). This is consistent with an earlier report by Higman et al. (1996), that saphenous veins from chronic smokers have decreased endothelial-dependent relaxation and NO synthesis and that pretreatment of these vein rings with BH_4 restores both of these deficits.

These observations are consistent with the finding that treatment of pulmonary artery endothelial cells with aqueous extracts of cigarette smoke lead to the time-dependent and irreversible inhibition of eNOS activity (Su et al., 1998). In another study, the treatment of rats with cigarette smoke caused a loss of constitutive NOS activity in the stomach, although the authors did not differentiate between eNOS or nNOS (Ma et al., 1999). In other studies, however, treatment of endothelial cells or aortas with cigarette smoke did not have an effect on eNOS activity (Raij et al., 2001). The exposure of rats to cigarette smoke causes the inactivation and loss of nNOS but not eNOS in penile tissue (Xie et al., 1997), suggesting isoform-selective inhibition in certain tissues. Recently, it was found with the use of an in vitro system containing purified proteins that water-soluble extracts made from cigarettes or cigarette smoke cause the direct inactivation of nNOS in a process that is metabolism- and time-dependent (Demady et al., 2003). Interestingly, excess L-arginine could protect nNOS from this inactivation, whereas excess BH_4 had no effect.

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ABBREVIATIONS: NO, nitric oxide; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; CSE, the water extract made from cigarette smoke; CE, the water extract made directly from the nonburned cigarette; BH_2, 7,8-dihydrobiopterin; HPLC, high-performance liquid chromatography; FT, flow through; SOD, superoxide dismutase; NONOate, diazenium diolate.

In the current study, we chose to examine whether the inhibition of eNOS occurs by a direct process similar to that observed with nNOS. Furthermore, we wanted to see whether the mechanism of inhibition could explain the isoform selectivity of the process as well as the NO and BH_4 deficit in smokers. We found, with the use of a purified system, that eNOS is inhibited in a time- and metabolism-dependent manner by water-soluble extract of cigarettes (CE) similar to that found for nNOS. However, unlike that found for nNOS, BH_4 reactivates eNOS after inhibition by CE, whereas L-arginine had no effect. The mechanism of inhibition involves the CE-dependent, eNOS-mediated oxidation of BH_4, leading to a BH_4-depleted enzyme. The direct action of chemical component(s) of cigarettes on eNOS may lead to new insights on the mechanism of eNOS catalysis and inhibition as well as in the better understanding of the action of smoking on vascular function.

Materials and Methods

Materials. L-[^{14}C(U)]Arginine (330.0 mCi/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) and 7,8-dihydro-L-biopterin were purchased from Schircks Laboratories (Jona, Switzerland). L-Arginine, naphthoquinone, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, calmodulin, catalase, superoxide dismutase, NADPH, and $NADP^+$ were purchased from Sigma-Aldrich (St. Louis, MO). Activated charcoal (Darco G60) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Regular nonfilter Camel cigarettes (R.J. Reynolds Tobacco Co.) were purchased from a local store. Research grade cigarettes (4A1 and 1R3) were purchased from the Tobacco and Health Research Institute, University of Kentucky (Lexington, KY).

Preparation of Cigarette Smoke Extract (CSE) and Cigarette Extract (CE). For preparation of cigarette smoke extracts, cigarette smoke was generated and bubbled through 10 ml of water at room temperature as described (Su et al., 1998). Each cigarette was burned over a 3- to 4-min duration with the use of a house vacuum line. Typically, 10 cigarettes were burned in series to make the stock solution of smoke extract, which was stored at 4°C. For preparation of cigarette extracts, four cigarettes were cut open, and the contents were ground with a mortar and pestle and subsequently transferred to a 50-ml plastic tube containing 40 ml of water. The tube was placed on a rotator mixer for 24 h at room temperature. The contents were passed through cheesecloth, and the liquid portion was spun at 2000*g* for 4 min. The supernatant was vacuum-filtered with the use of a glass-fitted filter, initially with a coarse mesh (40- to 60-μm) and then subsequently with a fine mesh (4- to 5.5-μm) filter. The clear solution was stored at 4°C for up to 1 month. The CSE and CE were prepared from 2 and 12 different batches of cigarettes, respectively. The CSE and CE preparations presented here are representative of all the batches in their ability to inhibit NOS.

Studies on the Nature of the eNOS Inactivator in CE. To test for volatility of the inactivator, we placed 3-ml aliquots of CSE or CE into glass vials, which were placed in a SpeedVac and dried to completeness (approximately 12 h). The residue was reconstituted in 3 ml of water and tested for the presence of the eNOS inactivator. This material was compared with the original solution of CSE or CE. We also determined whether the inactivator(s) would pass through a Centricon (3000 molecular weight cutoff; Millipore Corporation, Bedford, MA) filter. An aliquot (5 ml) of CE or CSE was placed in the Centricon filter and processed according to the manufacturer's instructions. The filter was spun at 3000*g* for 4 h at 4°C. The retentate was reconstituted to a final volume of 5 ml with water. The filtrate and retentate were tested for their ability to inactivate eNOS. The CE was also tested for binding to activated charcoal. A 250-μl aliquot of activated charcoal (5% w/v) was placed in an Eppendorf tube and spun (16,000*g*) on a microcentrifuge for 5 min. The water was taken off, and 500 μl of CE was added, and the tube was placed on a rotator for 30 min. The mixture was spun down again, and the supernatant was tested for the presence of the inactivator.

The nature of the inactivator(s) was also tested by ion-exchange chromatography. CE (15 ml) was loaded onto a cation exchange column (Bio-Rad AG 50W-X8, Bio-Rad, Hercules, CA; sodium form, 1 ml of resin loaded in a 5 3/4-inch disposable Pasteur pipette with a glass wool plug) equilibrated with water. Fractions were collected from the flow-through and tested for the presence of the inactivator. In other studies, CE was treated with an anion exchange column (Bio-Rad AG1-X10, chloride form) instead of the cation exchange column, and the resulting fractions were tested for the inactivator.

Treatment of NOS with CE and NOS Activity Assay. The rat nNOS and soluble eNOS containing a G_2A mutation were overexpressed in Sf9 insect cytosol and purified as previously described (Chen et al., 1995; Demady et al., 2003). The specific activities of nNOS and eNOS preparations were approximately 1050 nmol/min/mg protein and 40 nmol/min/mg protein, respectively, at 37°C.

Purified NOS (1.0 μM) was added to a "first reaction mixture" of 37 mM potassium phosphate, pH 7.4, containing 0.2 mM $CaCl_2$, 500 units/ml super-

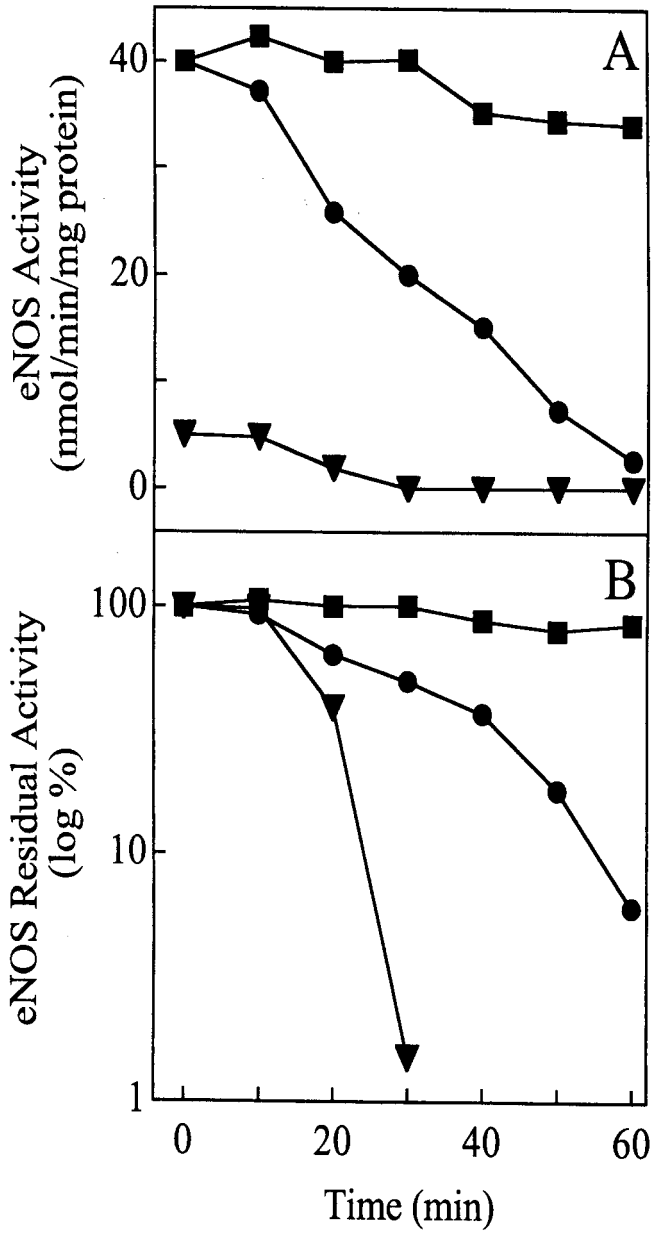

FIG. 1. Time-dependent inhibition of eNOS activity due to CE and CSE. The aqueous extracts made from smoke (CSE) or from nonburned Camel cigarettes (CE) were tested for time-dependent inhibition of eNOS activity. The loss of activity of eNOS was determined with the use of the first reaction mixture, and the oxyhemoglobin assay as described under *Materials and Methods*. A, the eNOS was incubated in the first reaction mixture in the absence of any extract (■), 0.015 cigarette equivalents of CE (●), or 0.06 cigarette equivalents of CSE (▼) and the eNOS activity was measured. B, data from A were replotted on a semilog plot. The figure presents one of two identical experiments.

oxide dismutase, 100 units/ml catalase, 80 μg/ml calmodulin, 2 μM BH_4, 1 mM dithiothreitol, the desired concentration of extract, and an NADPH-generating system composed of 0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase/ml, expressed as final concentrations, in a total volume of 180 μl. After incubation at 30°C, aliquots (15 μl) of the first reaction mixture were transferred to an "oxyhemoglobin assay mixture" containing 200 μM $CaCl_2$, 250 μM L-arginine, 100 μM BH_4, 100 units/ml catalase, 10 μg/ml calmodulin, 25 μM oxyhemoglobin, and an NADPH-generating system composed of 0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase/ml, expressed as final concentrations, in a total volume of 195 μl of 40 mM potassium phosphate, pH 7.4. The assay mixture was incubated at 37°C and the rate of NO-mediated oxidation of oxyhemoglobin was monitored with the use of a microtiter plate reader (SpectraMax Plus, Molecular Devices, Sunnydale, CA) as previously described (Feelisch et al., 1996). The rate was determined from the linear portion of the time-dependent changes in absorbance. In studies where NO synthesis was measured by the use of radiolabeled arginine, aliquots (20 μl) of the first reaction mixture were transferred to a "^{14}C-arginine assay mixture" containing 30 μM L-arginine (22 mCi/mmol), 1 mM $CaCl_2$, 100 μM BH_4, 100 units/ml catalase, 10 μg/ml calmodulin, and an NADPH-generating system composed of 0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase/ml, expressed as final concentrations, in a total volume of 150 μl of 20 mM Tris-Cl, pH 7.6. The assay mixture was incubated at 37°C for 10 min and the amount of radiolabeled citrulline was quantified.

Quantification of BH_4 and BH_2. The amounts of BH_4 and BH_2 in the reaction mixtures were determined by use of an HPLC fluorescence method as described by Klatt et al. (1996). The method involves oxidization of BH_4 and BH_2 to biopterin by treatment with KI/I_2 solution under acidic conditions. To give the specific amount of BH_2, the KI/I_2 oxidation is done in a basic solution where BH_4 and BH_2 are oxidized to pterin and biopterin, respectively. Specifically for oxidation under acidic conditions, a 5-μl aliquot of the first reaction mixture was treated with 10 mM I_2 and 50 mM KI in a total volume of 50 μl of 100 mM HCl for 1 h at room temperature in the dark. The solution was neutralized with 5 μl of 1.0 M NaOH, and then 5 μl of 0.2 M ascorbate was added. An aliquot (30 μl) of the resulting solution was injected onto a reverse phase HPLC column (C18 Vydac 5 mm, 4.6 × 250 mm) equilibrated with 20 mM NaH_2PO_4, pH 3, with 5% methanol at a flow rate of 1 ml/min. The pterins were eluted with the same mobile phase and detected by fluorescence at excitation and emission wavelengths of 350 and 418 nm, respectively. The HPLC and analysis of pterins were performed with the use of a Waters 600S system with a 717 autosampler (Waters, Milford, MA) and an Applied Biosystems Spectroflow 980 fluorescence detector (Applied Biosystems, Foster City, CA). To oxidize the pterins under basic conditions, the first reaction mixture was treated as above except that 100 mM NaOH replaced 100 mM HCl, and the final solution was neutralized with 5 μl of 1 M HCl.

Results

Water-Soluble CSE and CE Inhibit eNOS. Cigarettes were cut open and the contents were ground up and placed in water to obtain a preparation containing water-soluble compounds found in cigarettes (CE). As shown in Fig. 1A, treatment of eNOS with this CE solution causes a time-dependent loss of eNOS activity over that found in control samples (Fig. 1A, closed circles versus closed squares). The eNOS is nearly completely inactivated by 60 min. In another study, the cigarettes were burned and the smoke was bubbled through water to obtain a preparation of compounds that were extracted from the cigarette smoke (CSE). The CSE solution caused an immediate decrease in eNOS activity of approximately 85% and then a time-dependent loss of the remaining activity by 30 min (Fig. 1A, closed triangles). Clearly, for the CSE, the major effect was the immediate loss of activity, perhaps due to the presence of a reversible inhibitor. In the current study, however, we chose to focus on the time-dependent inhibition of eNOS, especially since this has been reported for nNOS (Demady et al., 2003). As shown in Fig. 1B, a replot of the data for the time-dependent inhibition on a log plot is nonlinear unlike that found for CE- and CSE-mediated inhibition of nNOS (Demady et al., 2003). There is an initial slow or lag phase(s) with a subsequent faster phase(s). We made no attempt at fitting or modeling these phases. As a control, we also measured eNOS activities by the ^{14}C-arginine assay and obtained highly similar results.

We compared the concentration-dependence of the CE on inactivating nNOS and eNOS at the 60-min time point (Fig. 2). The NOS activity is presented as a percentage of the control value, which is the activity found in the untreated sample after 60 min of incubation. The

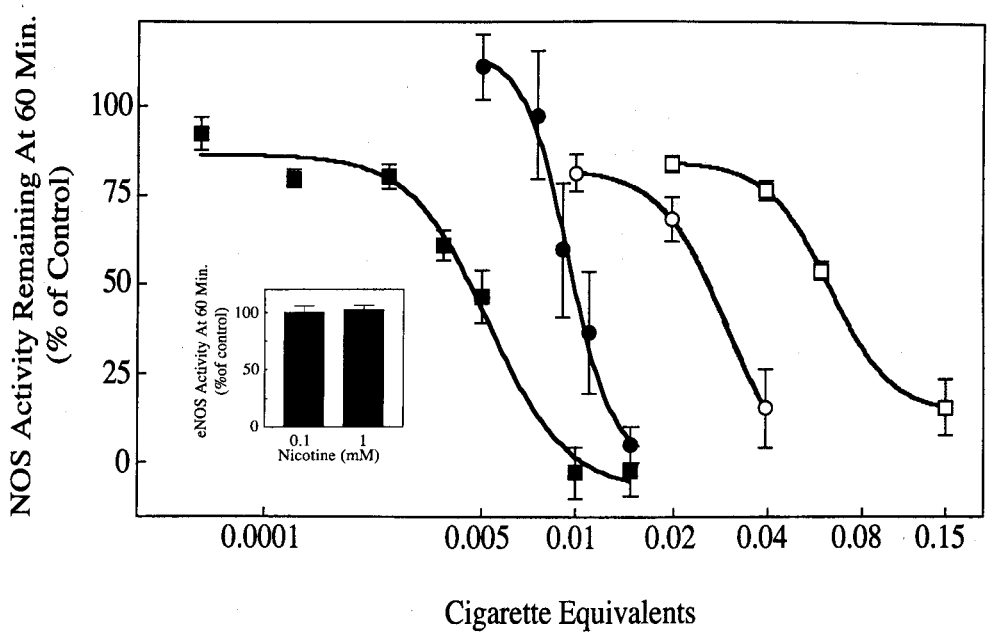

FIG. 2. The inhibition of eNOS and nNOS is dependent on the concentration of CSE or CE. The amount of NOS activity remaining was determined after 60-min incubation of CE with eNOS (●) and nNOS (■) and the incubation of CSE with eNOS (○) and nNOS (□) at the indicated concentrations. The NOS inhibition was determined using the first reaction mixture as described under *Materials and Methods*. The NOS activity remaining was determined by comparison to the activity remaining in the untreated (0 cigarette equivalents) condition after 60 min. The values are the mean of ± S.E. (n = 3). Inset, pure nicotine does not inactivate eNOS.

inhibition of nNOS by CE gave an IC_{50} of 0.0052 ± 0.0005 cigarette equivalents, which is comparable to that previously reported (Demady et al., 2003). The IC_{50} for eNOS was 0.0094 ± 0.0011 cigarette equivalents, but the dose-response curve was much steeper than for nNOS. The IC_{50} for CSE was 0.064 ± 0.004 cigarette equivalents and 0.030 ± 0.002 cigarette equivalents for nNOS and eNOS, respectively. As in the case of nNOS, nicotine up to a concentration of 1.0 mM did not inactivate eNOS (Fig. 2, inset). We also tested two types of research grade cigarettes made by the Tobacco and Health Research Institute, University of Kentucky. The CE prepared from the 1R3 and 4A1 batches were highly similar, with IC_{50} values of 0.0082 ± 0.0006 cigarette equivalents and 0.0072 ± 0.0004 cigarette equivalents. These values are similar to that obtained from the commercial cigarettes and suggest that additives or flavorings that may have been added in commercial preparations are not sources of the inhibitor. Although these values are useful when comparing the in vitro data, we do not know the correlation of these values relative to the actual human exposures during smoking.

Partial Characterization of the Inactivator in CE. We examined whether the components in the CE were similar in nature to that previously determined for inhibition of nNOS. To investigate whether the eNOS inactivator(s) is volatile, we placed CE under vacuum and took the sample to dryness by the use of a SpeedVac apparatus and then reconstituted back to the original volume with water. As shown in Fig. 3A, this reconstituted solution was able to inactivate eNOS (After) just as well as the original solution of CE that was not dried (Before), indicating that the compound(s) responsible for inactivating eNOS is nonvolatile. This indicates that the inactivator(s) is not carbon monoxide or NO, both agents known to inhibit eNOS. Next, we investigated whether the inactivator(s) is a high or low molecular mass compound(s). The CE was passed through a 3000 molecular weight-cutoff membrane, and the flow-through fraction (Filtrate) was found to contain the inactivator, whereas the retained fraction (Retentate), which was made to the original volume with water and tested, did not contain the inactivator. This indicates that the inactivator(s) has relatively low molecular mass and rules out most proteins. To examine whether the inactivator is an organic species, we treated CE with activated charcoal for 30 min, and the sample was spun and the supernatant was examined. We found that the inactivator(s) was bound to charcoal as judged by the lack of inhibition caused by the supernatant (Char). This suggests that the inactivator is an organic compound and not likely a free metal. To test for the ionic character of the inactivator(s), we tested for the ability of the inactivator(s) to bind to ion exchange resins. As shown in Fig. 3B, we found that the inactivator(s) in CE for eNOS was bound to a column containing a cation exchange resin as evidenced by the lack of inhibition caused by the flow-through fraction (condition 3, Cation, FT). This is entirely consistent with that found for nNOS with the same sample (condition 6, Cation, FT). However, some of the eNOS inactivator(s) did bind to an anion exchange resin (condition 2, Anion, FT) whereas very little of the nNOS inactivator(s) bound to the anion resin (condition 5, Anion, FT). The exact nature of the molecule(s) responsible for inhibition of eNOS or nNOS is unknown although the compounds appear to be organic, charged, nonvolatile, and of low molecular mass.

Effects of NADPH, Calmodulin, L-Arginine, Asymmetric Dimethyl-L-arginine, Glutathione, and BH_4 on the Time-Dependent Inhibition of eNOS by CE. To further investigate the nature of the time-dependent inhibition of eNOS, we added or deleted cofactors and substrates in the first reaction mixture (Fig. 4). The absence of NADPH (condition 2) or calmodulin (condition 3) either prevented or greatly hindered the inhibition due to CE, suggesting that a metabolically capable enzyme was needed for the inhibition to occur. This metabolic component is similar to that observed for nNOS (Demady et al., 2003). However, unlike for that found for nNOS, the inclusion of 0.1 mM L-arginine (condition 4, L-arg), or 0.1 mM asymmetric dimethyl-L-arginine (condition 5, ADMA), both compounds known to bind at the heme active site, did not protect eNOS from inhibition by CE. The inclusion of 10 mM glutathione (condition 6, GSH) had no effect on the inhibition. Interestingly, the inclusion of 100 μM BH_4 protects eNOS (condition 7) from CE-mediated inhibition, whereas BH_4 does not protect nNOS from CE-mediated inhibition (Demady et al., 2003).

We wondered how much BH_4 was needed to afford protection against the CE-mediated inhibition of eNOS and directly compared

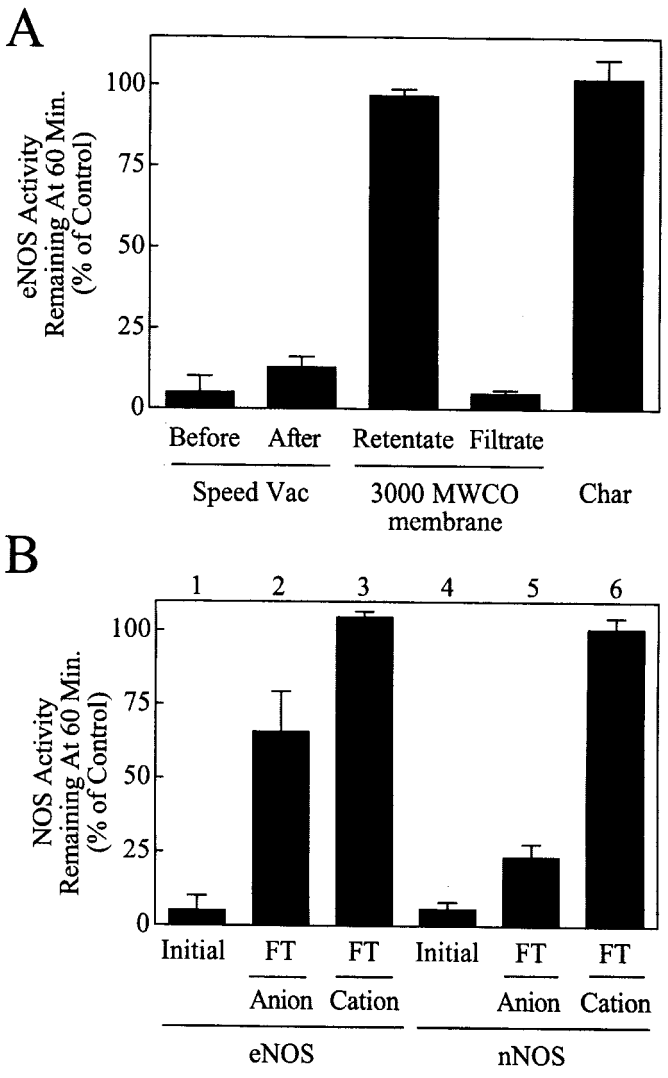

FIG. 3. Characteristics of the eNOS inactivator found in CE. A, effect of drying, ultrafiltration, or charcoal extraction on the presence of inactivator(s) in CE. The volatility of the inactivator(s) was tested by drying CE to completion in a SpeedVac. The presence of the inactivator(s) was tested before and after drying, as indicated. The ability of the inactivator(s) to pass through a Centricon 3000 molecular weight-cut-off (MWCO) membrane was also tested by measuring the extent of inhibition caused by the retentate and filtrate, as indicated. CE was treated with activated charcoal and then tested for effects on eNOS activity (Char). B, ability of the inactivator(s) in the cigarette extract (CE) to bind to a cation exchange resin or an anion exchange resin. CE was placed onto an anion or cation exchange resin and the flow-through (FT) fraction was prepared as described in *Materials and Methods*. The effect on eNOS and nNOS activity is shown for comparison. The effect of the initial CE preparation before treatment with resin is also shown (Initial). The values are the mean ± S.E. (n = 3).

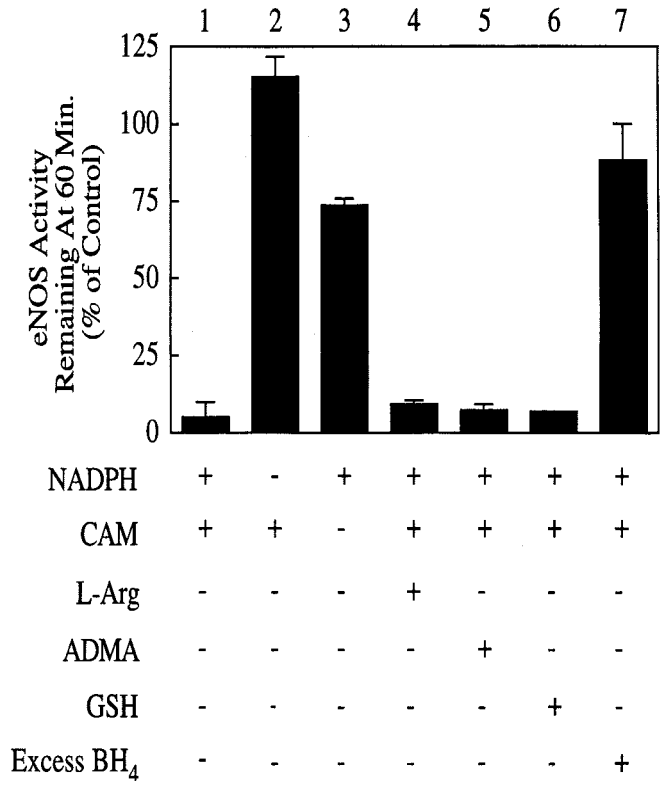

FIG. 4. The effect of NADPH, arginine, assymetric dimethyl-L-arginine, glutathione, and BH_4 on the inhibition of eNOS caused by cigarette extract. The eNOS (1 μM) was inactivated by CE (0.015 cigarette equivalents) for 60 min as described under *Materials and Methods* except that NADPH-generating system (NADPH), calmodulin (CAM), 0.1 mM L-arginine (L-arg), 0.1 mM asymmetric dimethyl-L-arginine (ADMA), 10 mM glutathione (GSH), and 100 μM BH_4 (Excess BH_4) were added or deleted from first reaction mixture, as indicated. There is 2 μM BH_4 in the first reaction mixture except in the case of "excess BH_4", where 100 μM BH_4 is present. The values are the mean ± S.E. (n = 3).

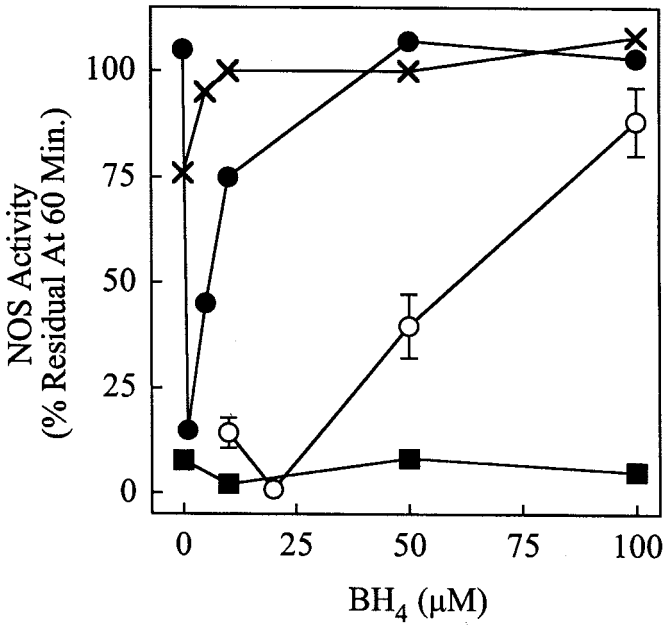

FIG. 5. . The effect of BH_4 on the CE-mediated inhibition of eNOS and nNOS. The eNOS (●) or nNOS (■) was treated with CE (0.015 cigarette equivalents) for 60 min as described under *Materials and Methods* except that the amount of BH_4 added to the first reaction mixture was varied, as indicated. In the case of eNOS, CE was omitted from the first reaction mixture (X) in some studies. The values are for single experiments. In another experiment for eNOS, the oxyhemoglobin assay mixture did not contain any exogenous BH_4 and contained only that brought over from the first reaction mixture (O). The values for this study are the mean ± S.E. (n = 3).

that to nNOS (Fig. 5). In the case of eNOS, the greatest CE-mediated inhibition was observed at 1 μM BH_4 with higher concentrations giving a concentration-dependent protection against inhibition with complete protection at 50 μM BH_4 (Fig. 5, closed circles). Interestingly when BH_4 is not added to the reaction mixture, there is no inhibition, presumably because the eNOS is inactive or does not stay active and thus cannot carry out the metabolic step necessary for CE-mediated inhibition to occur. This notion is consistent with the lack of eNOS inhibition in the absence of NADPH or calmodulin seen above. There is a protective effect of BH_4 from the slight autoinactivation of eNOS seen in the absence of CE (X). In contrast to that found for the CE-mediated inhibition of eNOS, the inhibition of nNOS occurs at concentrations of BH_4 above 1 μM, and no protection is observed (Fig. 5, closed squares). Thus, the mechanism of CE-mediated inhibition of eNOS appears distinct from that of nNOS. In these studies, we changed the concentration of BH_4 in the first reaction mixture but had 100 μM BH_4 in the subsequent oxyhemoglobin assay mixture used for measuring the eNOS activity. In a separate study, we omitted the BH_4 present in the oxyhemoglobin assay mixture and discovered that the inhibition was greatly sensitized to concentration of BH_4 under these conditions (Fig. 5, open circles). This suggested to us that perhaps BH_4 was reactivating the eNOS during the oxyhemoglobin assay and this notion was tested as described below.

BH_4 Reverses the CE-Mediated Inhibition of eNOS. As shown in Fig. 6A, eNOS was first inactivated with CE for 60 min and then 100 μM BH_4 was added, and aliquots were taken over time to assess enzyme activity. The inhibition of eNOS was nearly complete under these conditions, but the activity slowly increased over the next 10 min, ultimately recovering approximately 75% of the total. The recovery of the activity was dependent on the concentration of BH_4 in the reaction mixture during the 10-min recovery period (Fig. 6B). This effect was not due to dithiothreitol, which is present in the stock solution of BH_4 (Fig. 6C). The inhibition is reversible with BH_4 and the reversal suggests that pterin depletion is the mechanism of inhibition.

The eNOS Metabolizes BH_4 during the Treatment with CE. To further understand the role of BH_4 in the inhibition, we examined whether BH_4 is indeed lost during the inhibition reaction with eNOS. To do this, the starting reaction mixture was treated with KI/I_2 solution under basic conditions to oxidize the starting BH_4 to pterin, which is subsequently detected by an HPLC fluorescence method (Fig. 7A, peak 1). After incubation for 60 min, the eNOS reaction mixture is submitted to the same analysis and found to contain not pterin but biopterin (Fig. 7A, peak 2), which is the oxidation product of BH_2. As shown in Fig. 7C, we quantified both products and found that during the inhibition of eNOS, the loss of BH_4 (solid squares) is concomitant to the appearance of BH_2 (solid circles). Moreover, the loss of BH_4 during the inhibition of eNOS is dependent on the presence of CE and calmodulin (Fig. 8, left panel). Interestingly, the inhibition of nNOS by CE is not associated with a marked decrease in BH_4 (Fig. 8, right panel). Thus, nNOS is inactivated by CE by a different mechanism that does not involve the depletion of BH_4.

In all previous studies above, the first reaction mixtures contained 500 units/ml SOD and 100 units/ml catalase to minimize the effect of reactive oxygen species that are produced by eNOS. This was a concentration of both enzymes that was effective in protecting against oxidative damage of nNOS (Demady et al., 2001, 2003). Based on the

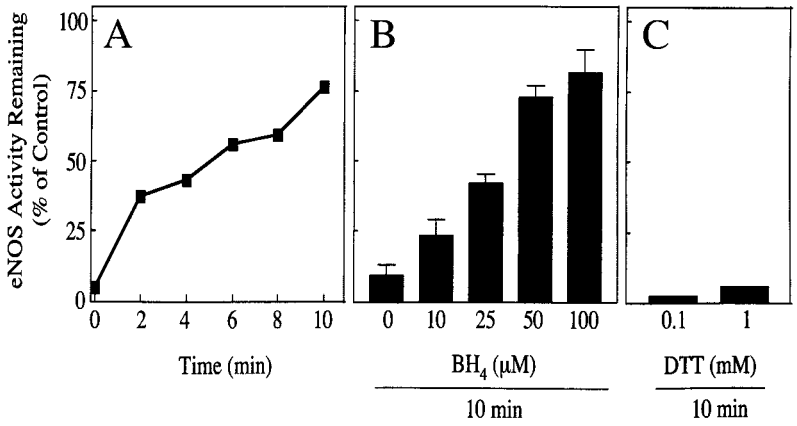

FIG. 6. BH_4 reactivates CE-treated eNOS in a time- and concentration-dependent manner. The eNOS was inactivated with CE and then incubated with BH_4, and the eNOS activity was determined. Purified eNOS (1 μM) was incubated for 60 min in the first reaction mixture containing 10 μM BH_4. A, time-dependence of the reactivation with 100 μM BH_4. The BH_4 was added to the CE-inactivated eNOS at time 0, and eNOS activity was measured over the indicated time intervals. B, the dependence on the concentration of BH_4 used for reactivation was determined. CE-inactivated eNOS was reactivated for 10 min in the presence of the indicated concentrations of BH_4. C, the CE-inactivated eNOS was treated for 10 min in the presence of the indicated amounts of dithiothreitol (DTT) as a control. The BH_4 stock solutions contained equimolar amounts of DTT, which was added for stability purposes. The values in B and C are the mean ± S.E. (n = 3). The values in A are from one experiment.

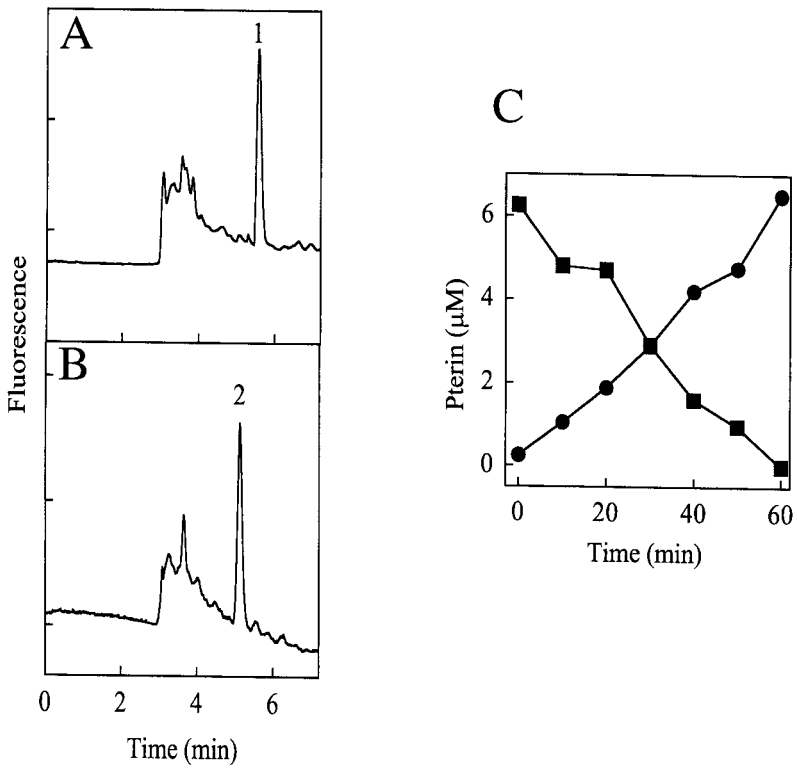

FIG. 7. There is a loss of BH_4 during the incubation of eNOS with CE. The eNOS was treated with 0.015 CE as described under *Materials and Methods* except that 6.5 μM BH_4 was added. The amount of BH_4 and BH_2 in the eNOS reaction mixture was quantified by HPLC. Peak 1 corresponds to pterin, and peak 2 corresponds to biopterin. A, HPLC profile of the reaction mixture immediately after addition of CE; B, HPLC profile of the reaction mixture 60 min after addition of CE; C, the amount of BH_4 (■) and BH_2 (●) present in the reaction mixture were quantified. The time represents the duration of exposure of eNOS to CE in the reaction mixture. The values are from a single experiment.

finding that 5000 units/ml SOD was needed in a study on the mechanism of BH_4-induced oxidation of NO with nNOS (Mayer et al., 1995), a more detailed analysis of the dependence on catalase and SOD was performed (Fig. 9). Higher concentrations of SOD were found to protect against the loss of BH_4 with nearly complete protection at 5000 units/ml (Fig. 9, solid circles). Catalase at concentrations up to 1000 units/ml had no effect (Fig. 9, solid triangles). In a previous study, 100 units/ml catalase completely protected nNOS from the oxidative inactivation caused by agmatine (Demady et al., 2001). SOD alone was nearly as effective as SOD in combination with catalase (Fig. 9, solid squares). Thus, it appears that the eNOS-mediated superoxide formation is mainly responsible for the oxidation of BH_4 caused by CE.

Discussion

We have shown for the first time that some chemical component(s) of cigarettes (CE) directly inhibits eNOS. This inhibition is due to the CE-mediated, eNOS-catalyzed oxidation of BH_4 that in turn leads to

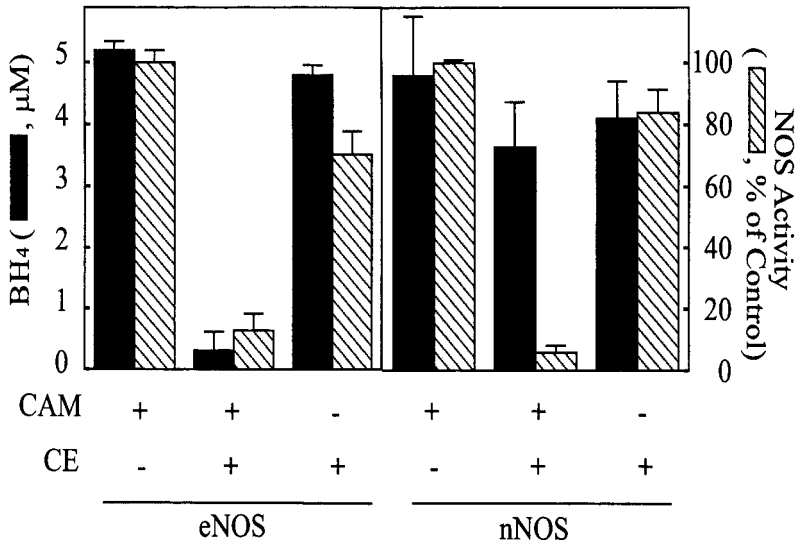

FIG. 8. The loss of BH_4 is dependent on calmodulin and occurs more readily with eNOS than with nNOS. The nNOS or eNOS was treated with CE for 60 min as in Fig. 7, and the NOS activity and pterin content of the reaction mixtures were measured. Where indicated, the calmodulin (CAM) and cigarette extract (CE) were omitted from the reaction mixtures. The values are the mean ± S.E. (n = 3).

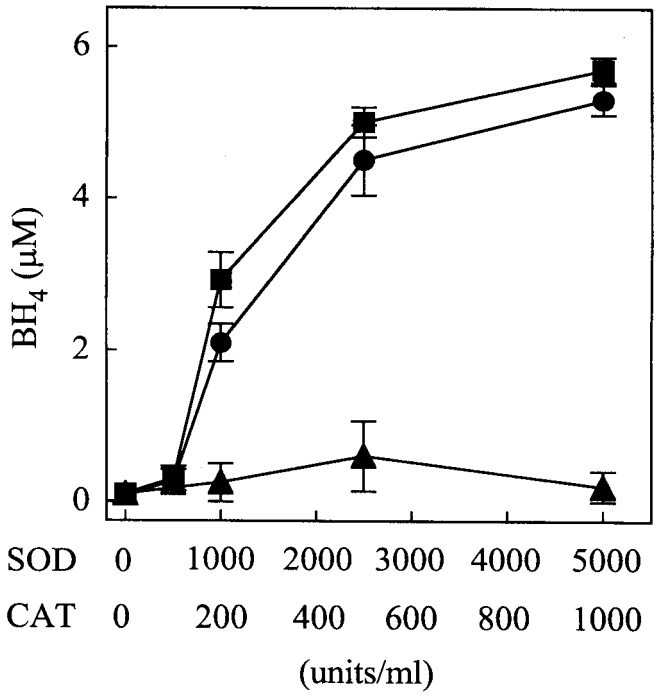

FIG. 9. Superoxide dismutase but not catalase prevents the oxidation of BH_4 catalyzed by eNOS. The eNOS was treated with CE for 60 min as in Fig. 7, and the NOS activity and pterin content of the reaction mixtures were measured. The amount of SOD (closed circles), catalase (CAT; closed triangles), and the combination of SOD and catalase (closed squares), were varied in the first reaction mixture and the effects on pterin were measured. The values are the mean ± S.E. (n = 3).

a BH_4-deficient eNOS that is unable to produce NO. This mechanism is consistent with the observed time-, NADPH-, and calmodulin-dependence of the inhibition caused by CE. Moreover, excess BH_4 not only protected eNOS from inhibition but also completely reactivated the enzyme after treatment with CE. Although eNOS inhibition by CE is highly sensitive to the amounts of BH_4 present, L-arginine had no effect. Interestingly, CE also inactivates nNOS in a metabolism- and time-dependent manner, but BH_4 does not protect or reactivate nNOS, whereas L-arginine does protect. Clearly, the mechanisms of inhibition of nNOS and eNOS by CE are distinct. Since we have not been able to isolate and identify the chemical(s) responsible for inhibition of the enzymes, the reason for these differences is unclear. It may be that different chemicals are responsible for inactivation of nNOS and eNOS or that one chemical acts differently upon these isoforms. In either case, the chemicals appear to be cationic, low molecular mass, nonvolatile, organic molecules.

Although it is possible that reactive chemicals present in CE, such as peroxynitrite (Muller et al., 1997), could oxidize BH_4, we have found that incubation of CE with BH_4 in the absence of eNOS does not result in the loss of BH_4. Furthermore, the oxidation of BH_4 requires not only CE but the presence of eNOS, Ca^{2+}-calmodulin, and NADPH, clearly indicating the need for an active eNOS in the process. A variety of NOS products, including NO, oxidants, and peroxynitrite, are known to cause the loss of BH_4 (Mayer et al., 1995; Reif et al., 1999; Witteveen et al., 1999; Werner-Felmayer et al., 2002). CE likely contains L-arginine, and thus NO may be produced, but L-arginine alone does not inhibit eNOS under our conditions. Asymmetric dimethyl-L-arginine did not protect from CE-mediated eNOS-inhibition, further supporting the notion that metabolism of L-arginine in CE likely does not play a role. Furthermore, Reif et al. (1999) reported that the consumption of BH_4 by nNOS during catalysis or by treatment with spermine NONOate alone does not give rise to BH_2 or biopterin. Since we have found the BH_4 is oxidized to BH_2 and biopterin, the reaction observed in our study appears to be distinct from the NO-mediated reaction observed by Reif et al. (1999). Thus, it is unlikely that NO formation alone or in combination with oxidant factors in CE explains the loss of BH_4. Based on the ability to protect BH_4 from oxidation by SOD, the eNOS-mediated formation of superoxide appears to be the most likely mechanism. We speculate that some redox active chemical(s) present in cigarettes leads to oxidative uncoupling of eNOS. A similar calmodulin-dependent, metabolism-based redox cycling of nNOS is caused by a variety of quinones (Kumagai et al., 1998). The quinone-dependent redox cycling also occurs with eNOS (Vasquez-Vivar et al., 1997). Moreover, redox active quinones are present in the aqueous extracts of cigarettes

(Winston et al., 1993) and tobacco leaves (Khalil et al., 2000). We do not know why greater BH_4 oxidation is catalyzed by eNOS over that found for nNOS during treatment with CE.

There are over 5000 chemicals characterized in tobacco, and we have attempted to purify the active ingredient(s) by organic solvent extraction, ion-exchange chromatography, and reverse phase chromatography. Although we have ruled out nicotine and NO, to date we have not succeeded in identifying the inactivator(s). We have also tested naphthoquinone at concentrations up to 1 mM and have found less than 10% inactivation after 60 min of treatment. Thus, further studies are needed on the nature of the inhibitor present in the cigarette as well as in the cigarette smoke. Indeed, the effects of the smoke are much more complex, with a time-dependent inactivator as well as an immediately acting inhibitor, perhaps a competitive inhibitor. There may actually be many inactivators present or even several structurally related inactivators that may greatly complicate the identification of the active components. We will aim to use LC-MS technology along with a high-throughput activity assay to better handle complex mixtures derived from cigarettes.

It is thought that smoking elicits a general oxidative stress leading to the loss of BH_4 that is ameliorated by antioxidants or administration of excess BH_4 (Ota et al., 1997; Heitzer et al., 2000). The oxidative stress may arise from redox reactions catalyzed by a variety of enzymes, including NADPH oxidase (Landmesser et al., 2003) and xanthine oxidase (Guthikonda et al., 2003). We show here that chemicals found in cigarettes can directly affect eNOS to oxidize BH_4, and we believe that the direct action of cigarettes on eNOS may play a role in the BH_4 deficit observed in smokers. Although both eNOS and nNOS are inhibited by CE, BH_4 reactivates eNOS but not nNOS, whereas L-arginine protects nNOS but not eNOS. Thus, differential effects might be expected and this may be the basis for isoform selective inhibition of nNOS over eNOS in penile tissue from rats treated with cigarette smoke (Xie et al., 1997). Certainly, the direct effect of chemicals in cigarettes on NOS should be considered as a mechanism to be assessed in future studies.

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Address correspondence to: Yoichi Osawa, Department of Pharmacology, University of Michigan Medical School, 1301 Medical Science Research Building III, Ann Arbor, MI 48109-0632. E-mail: osawa@umich.edu