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# METABOLISM-BASED INACTIVATION OF NEURONAL NITRIC-OXIDE SYNTHASE BY COMPONENTS OF CIGARETTE AND CIGARETTE SMOKE

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**ABSTRACT:**

**It has been shown that administration of cigarette smoke to rats leads to loss of neuronal nitric-oxide synthase (nNOS) activity and nNOS protein in penile tissue. The exact mechanism for this loss of activity and protein is not known. In the current study, we investigated whether extracts prepared from cigarette smoke or from the cigarette itself could directly inhibit nNOS activity. We discovered that the cigarette smoke extract and the cigarette extract cause a time-, concentration-, and calmodulin-dependent inactivation of nNOS in an in vitro system containing the purified enzyme. L-Arginine, but not D-arginine, protects nNOS from this time-dependent inactivation, suggesting an active site directed event. The kinetics of inactivation are consistent with the metabolism-based or suicide inactivation of nNOS. Based on studies with other metabolism-based inactivators, this cigarette-mediated inactivation may render nNOS more susceptible to proteasomal degradation and thereby may explain the loss of nNOS protein in vivo. The component(s) responsible for nNOS inactivation is not volatile, is not retained by a 3,000 molecular weight cut-off membrane, binds to activated charcoal, and is highly water-soluble under both acidic and basic conditions. The discovery of a direct inactivation of nNOS by an organic, cationic compound(s) present in tobacco and tobacco smoke provides a basis for further study of not only the mechanisms responsible for the biological effects of tobacco but also a search for a potentially novel inactivator of nNOS.**

Cigarette smoking in man is associated with endothelial dysfunction and impotence and since NO[3] is known to play a central role in the physiological regulation of blood pressure and penile erection, it is thought that a decreased capability to produce NO plays a central role in these pathological conditions (Rosen et al., 1991; Shabsigh et al., 1991; Mannino et al., 1994; Xie et al., 1997; Heitzer et al., 2000). Moreover, cigarette smoking reduces exhaled NO in man, an indicator of decreased endogenous NO production (Kharitonov et al., 1995). This endogenous production of NO is due to NO synthase (NOS), a hemoprotein enzyme that catalyzes the conversion of L-arginine to citrulline and NO. Three main isoforms of NOS have been identified: isoform I (nNOS), which is constitutively expressed in a variety of neuronal cells; isoform II, which is usually not constitutively expressed but can be induced by bacterial lipopolysaccharide and/or cytokines in macrophages; and isoform III (eNOS), which is expressed in endothelial cells (Lancaster and Hibbs, 1990; Forstermann et al., 1994). The enzymes also require bound heme, FMN, FAD, tetrahydrobiopterin, and $Ca^{+2}$/calmodulin for activity (Marletta, 1993).

The inhibitory effects of cigarette constituents and cigarette smoke on biologically available NO have also been extended to cellular and animal models. Extracts of cigarette smoke cause a time- and dose-dependent loss of eNOS activity and eNOS protein in pulmonary artery endothelial cells (Su et al., 1998). Exposure of rats to cigarette smoke causes a decrease in nNOS activity and nNOS protein in penile tissue while leaving eNOS protein unchanged (Xie et al., 1997). A loss of nNOS protein in rat brainstem was observed after prenatal exposure to cigarette smoke (Hasan et al., 2001), although no loss of nNOS protein was found in adult rat lung (Wright et al., 1999). Thus, it appears that smoking can lead to a selective loss of NOS activity and protein depending on the conditions and tissues; however, the mechanism by which this activity and protein are lost is not known.

We wondered if there is a direct interaction of some of the chemicals present in cigarettes with NOS. In the current study, we examined whether chemicals found in cigarette smoke or in the cigarette itself could directly inhibit nNOS and the mechanism by which this occurs. We have discovered, with the use of an in vitro system containing purified nNOS, that aqueous extract of cigarette or cigarette smoke inactivates nNOS in a time- and metabolism-based manner. The active entity is a low molecular weight, charcoal-extractable, water-soluble, nonvolatile compound(s). The compound is not nicotine. In that other metabolism-based inactivators of nNOS have been shown to lead to enhanced proteasomal degradation of nNOS (Osawa et al., 2003), the inactivation of the enzyme by cigarette constituents

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[2] Trainee under Pharmacological Sciences Training Program GM07767 from the National Institutes of Health.

[3] Abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; $BH_4$, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; CSE, the water extract made from cigarette smoke; CE, the water extract made directly from the nonburned cigarette; CAM, calmodulin; ASE, Accord smoke extracts; LSE, *Lattuca sativa* smoke extracts; ADMA, asymmetric dimethyl-L-arginine.

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in a metabolism-based manner may explain the loss of nNOS protein associated with inhalation of cigarette smoke. Thus, this discovery of a direct mechanism of inactivation of nNOS and the nature of the cigarette constituents responsible for this inactivation may aid in understanding some of the biochemical changes associated with smoking.

## Materials and Methods

**Materials.** L-[$^{14}$C(U)]Arginine (330.0 mCi/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin ($BH_4$) was purchased from Dr. Schirck's Laboratory (Jona, Switzerland). L-Arginine, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, calmodulin, catalase, NADPH, and $NADP^+$ were purchased from Sigma-Aldrich (St. Louis, MO). Activated charcoal (Darco, G60) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Methylene chloride was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Regular nonfilter Camel cigarettes (R. J. Reynolds Tobacco Co.) were purchased from a local store. Accord cigarettes (Philip Morris, Inc.) and Bravo cigarettes (cigarettes prepared from Latucca sativa, nicotine free; Bravo Smokes Inc., Hereford, TX) were obtained from Drs. Bert La Du and Ed Domino, respectively. Research grade cigarettes (4A1 and 1R3) were purchased from the Tobacco and Health Research Institute, University of Kentucky (Lexington, KY).

**Methods.** *Preparation of cigarette smoke extract (CSE) and cigarette extract (CE).* For preparation of CSE, 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. In the case of Accord cigarettes, the same vacuum apparatus was used, but the cigarettes were placed in the heating apparatus as instructed by the manufacturer to generate the smoke. For preparation of CE, 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 2,000*g* for 4 min. The supernatant was vacuum-filtered with the use of a glass-fritted filter, initially with a coarse mesh (40 to 60 $\mu$m) and then subsequently with a fine mesh (4 to 5.5 $\mu$m) filter. The clear solution was stored at 4°C.

*Treatment of CSE and CE.* To determine the acid/base characteristics of the inactivator, a series of extractions with organic solvent under acidic and basic conditions was performed. A solution of HCl (6 N) was added to 50 ml of CSE or CE until the pH was approximately 2. This solution was subsequently extracted with an equal volume of methylene chloride. The organic fraction was further extracted with an equal volume of 1 N NaOH to give an organic fraction (Fraction 1) and an aqueous fraction (Fraction 2). The aqueous fraction from the original acidic extraction was made basic (pH 10) with 2 N NaOH and then extracted with an equal volume of methylene chloride. This gave an organic fraction (Fraction 3) and an aqueous fraction (Fraction 4). The organic fractions were dried with $Na_2SO_4$, flash evaporated, and the residue brought up in 1 ml of ethanol and stored at 4°C. The aqueous fractions were immediately neutralized with 6 N HCl, put under a vacuum to remove residual methylene chloride, and stored at 4°C. For testing in nNOS assays described below, the ethanol solution was added to glycerol (10% as a final concentration in the reaction mixture) and the ethanol removed by a gentle stream of nitrogen.

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 (Thermo Savant, Holbrook, NY), and dried to completeness (approximately 12 h). The residue was reconstituted in 3 ml of water and tested for its ability to inactivate nNOS as described below. We also determined whether the inactivator(s) would pass through a Centricon (Amicon, 3,000 molecular weight cut-off; Millipore Corporation, Bedford, MA) filter. A 5-ml aliquot of CE or CSE was placed in the Centricon filter and processed according to the manufacturer's instructions. The filter was spun at 3,000*g* for 4 h at 4°C. The retentate was reconstituted to a final volume of 5 ml with water. An aliquot (144 $\mu$l) of the filterate, retentate, or starting material was tested for its ability to inactivate nNOS by the use of the oxyhemoglobin assay described below.

The nature of the inactivator(s) was also tested by ion-exchange chromatography. CE (1 ml) was loaded onto a cation exchange column (Bio-Rad AG 50W-X8, sodium form, loaded in a 5 3/4 inch disposable pasteur pipette with a glass wool plug; Bio-Rad, Hercules, CA) equilibrated with water. The column was washed with water (2 ml) and then washed with 2M NaCl (1 ml). Fractions were collected from the flow-through, water wash, and salt wash steps. In other studies, CE was treated with an anion exchange column (Bio-Rad AG1-X10, chloride form) instead of the cation exchange column. The CE was also tested for binding to activated charcoal. An aliquot (250 $\mu$l) of a mixture 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 $\mu$l 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.

*Expression and purification of nNOS.* nNOS was overexpressed in Sf9 insect cells as previously described (Bender et al., 1999). Oxyhemoglobin (25 $\mu$M) was added as a source of heme during expression. Cells were harvested and suspended in 1 volume of 10 mM Hepes, pH 7.5, containing 320 mM sucrose, 100 $\mu$M EDTA, 0.1 mM dithiothreitol, 10 $\mu$g/ml trypsin inhibitor, 100 $\mu$M leupeptin, 2 $\mu$g/ml of aprotinin, 6 mM phenylmethanesulphonyl fluoride, and 10 $\mu$M $BH_4$, and the suspended cells were ruptured by Dounce homogenization. Lysates from infected Sf9 cells ($8 \times 10^9$) were centrifuged at 100,000*g* for 1 h. The supernatant fraction was loaded onto a 2′5′-ADP Sepharose column (8 ml), and the nNOS was affinity purified as described (Roman et al., 1995), except that 10 mM 2′-AMP in high salt buffer was used to elute the protein. The nNOS-containing fraction was concentrated with the use of a Centriplus YM-10 concentrator (Amicon, 10,000 molecular weight cut-off; Millipore Corporation) to 10 ml and loaded onto a Sephacryl S-300 HR gel filtration column (2.6 × 100 cm; Amersham Biosciences Inc., Piscataway, NJ, equilibrated with 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5 $\mu$M $BH_4$. The proteins were eluted at a flow rate of 1.0 ml/min, and 1.0-ml fractions were collected and analyzed for protein content and NOS activity. The fractions containing NOS activity were pooled and supplemented with 10 $\mu$M $BH_4$ and concentrated with the use of a Centriplus YM-10 concentrator (Millipore Corporation). This Sephacryl-purified nNOS preparation has a specific activity of 1150 nmol/min/mg of protein and was stored at −80°C.

*Treatment of nNOS with inactivator(s) and nNOS activity assay.* Purified nNOS (80 $\mu$g/ml) was added to a "first reaction mixture" of 40 mM potassium phosphate, pH 7.4, containing 0.2 mM $CaCl_2$, 500 unit/ml superoxide dismutase, 100 units/ml catalase, 80 $\mu$g/ml crude calmodulin, 100 $\mu$M $BH_4$, 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 $\mu$l. In control samples, we have omitted the cigarette extract from the first reaction mixture described above. After incubation at 30°C, aliquots (10 $\mu$l) of the first reaction mixture were transferred to an "oxyhemoglobin assay mixture" containing 200 $\mu$M $CaCl_2$, 250 $\mu$M L-arginine, 100 $\mu$M $BH_4$, 100 units/ml catalase, 10 $\mu$g/ml crude calmodulin, 25 $\mu$M 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 190 $\mu$l 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 by measuring the absorbance at λ401 nm − 411 nm with a microtiter plate reader (SpectraMax Plus, Molecular Devices Corp., Sunnyvale, CA). 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 (10 $\mu$l) of the first reaction mixture were transferred to a "$^{14}$C-arginine assay mixture" containing 100 $\mu$M L-arginine (22 mCi/mmol), 200 $\mu$M $CaCl_2$, 100 $\mu$M $BH_4$, 100 units/ml catalase, 10 $\mu$g/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 100 $\mu$l of 40 mM potassium phosphate, pH 7.4. The assay mixture was incubated at 37°C for 5 min, and the amount of radiolabeled citrulline was quantified.

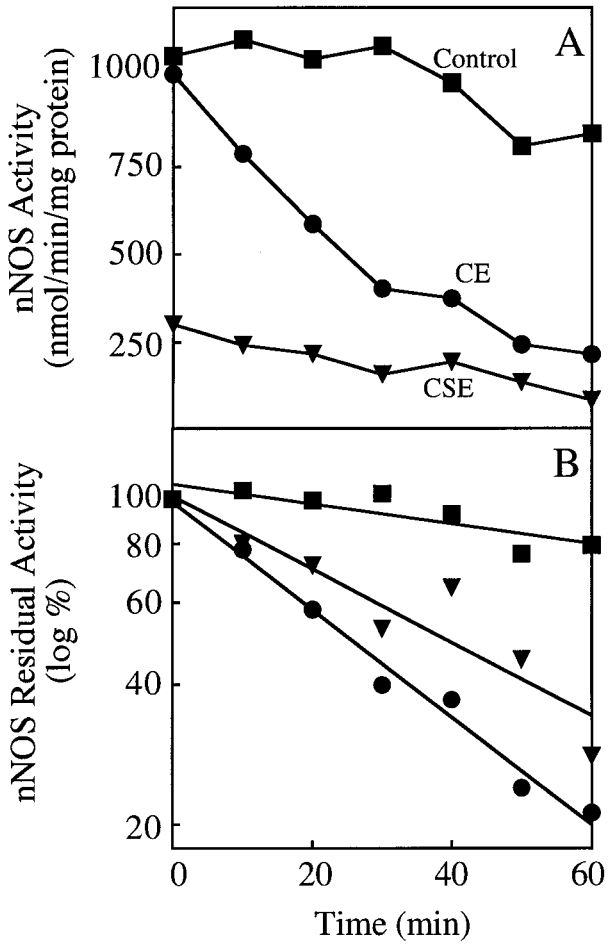


FIG. 1. *Time-dependent inactivation of nNOS by CSE and CE.*

The aqueous extracts made from smoke or from nonburned Camel cigarettes were tested for time-dependent inactivation of nNOS activity. The loss of nNOS activity was determined with the use of the "first reaction mixture" and the oxyhemoglobin assay as described under *Materials and Methods*. A, The nNOS was incubated in the first reaction mixture in the absence of any extract (■), 0.015 cigarette equivalents of CE (●), or 0.15 cigarette equivalents of CSE (▼). B, the time-dependent loss of activity in A is presented on a semi-log plot with the time 0 value as 100%.

## Results

**Water-Soluble CSE and CE Inhibit nNOS.** As shown in Fig. 1A, treatment of nNOS with the water-soluble extract made from cigarettes (CE) caused a time-dependent loss of nNOS activity with nearly 75% decrease in 60 min over that found in control samples (closed circles versus closed squares). The control samples were obtained by omitting the CE from the reaction mixture. The approximately 20% loss of activity in these control samples reflects the autoinactivation of the enzyme under the assay conditions. CSE caused an immediate decrease in nNOS activity of approximately 70% and then a time-dependent decrease in activity over the next 60 min (closed triangles). The loss of activity is presented on a semi-log plot in Fig. 1B with the activity of the sample at "zero" time set to 100%, to further illustrate the nature of the loss of activity that is time-dependent. In the current study, we chose to focus on this time-dependent inactivation of nNOS. We therefore determined the residual nNOS activity at 60 min and expressed the results as a percentage of that found in the control sample as a way to quantify the amount of time-dependent inactivation that occurred. As shown in Fig. 2A, this time-dependent loss in activity is dependent on the amount of CSE added, with nearly 90% inactivation at 0.15 cigarette equivalents. This loss of activity was entirely dependent on the presence of calmodulin in the first reaction mixture (CAM). Similarly, the time-dependent loss of activity seen with CE was also dependent on the concentration of extract in the first reaction mixture (Fig. 2B) with 50% inactivation seen at 0.005 cigarette equivalents, which is approximately 10-fold lower than that for CSE. This is likely due, in part, to the extraction efficiency of the process used to make CSE and CE. CE is prepared by extraction of cigarette for many hours whereas CSE is made by rapidly bubbling cigarette smoke into water. The inactivation due to CE, as in the case of CSE, was also dependent on calmodulin (CAM). As shown in Fig. 2C, the water-soluble smoke extract was prepared from Accord cigarettes (ASE) and from cigarettes made from *Lattuca sativa*, a variant of lettuce (LSE) and tested for time-dependent inactivation. The ASE extract gave weak inactivation whereas no inactivation was seen for the LSE extract at a concentration of 0.06 cigarette equivalents. The extract made directly from the Accord cigarettes caused approximately 99% loss of activity at 60 min at a dose of 0.06 equivalents. In future studies, we chose to focus on the effect of CE as the time-dependent loss in activity was seen independent of the immediate inhibition of the enzyme.

**Effects of NADPH, Arginine, and Asymmetric Dimethyl-L-arginine ($N^G$,$N^G$-dimethyl-L-arginine) on the Time-Dependent Inactivation of nNOS by CE.** To further investigate the nature of the time-dependent inactivation of nNOS, we added or deleted cofactors and substrates in the first reaction mixture (Fig. 3). The absence of calmodulin (condition 2) or NADPH (condition 3) prevented the inactivation due to CE, suggesting that a metabolically capable enzyme was needed for the inactivation to occur. The inclusion of L-arginine (condition 4), but not D-arginine (condition 5), protected the enzyme from inactivation due to CE suggesting that the inactivation is an active site-directed process. Furthermore, asymmetric dimethyl-L-arginine (ADMA, condition 6), which is a competitive NOS inhibitor acting on the substrate binding site, could also protect the enzyme from inactivation. A competitive inhibitor will decrease the metabolism of the mechanism-based inactivator and since metabolic activation of the inactivator is necessary for formation of the reactive intermediate, which inactivates the enzyme, the overall effect should be protection of the enzyme by the inhibitor.

**Partial Characterization of the Inactivator in CE.** To investigate whether the nNOS 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 the sample with water. As shown in Table 1, this SpeedVac evaporated and then reconstituted sample was able to inactivate nNOS just as well as the original solution of CE that was not dried (none), indicating that the compound(s) responsible for inactivating nNOS is nonvolatile. Next, we investigated if the inactivator(s) is a high or low molecular mass compound(s). The CE was passed through a 3,000 molecular weight cut-off 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) is a compound(s) of relatively low molecular mass. As shown in Table 1, the CE was extracted with methylene chloride under acidic and basic conditions to generate four fractions. These fractions were assessed for the presence of the inactivator(s) and compared with the original starting material. As might be expected from the water soluble-nature of the compounds, nearly all the inactivator(s) remained in the aqueous phase throughout the extraction procedure and ended up in Fraction 4. As shown in Table 1, we also found that the inactivator(s) in CE was bound to a column containing a cation exchange resin as evidenced by the lack of

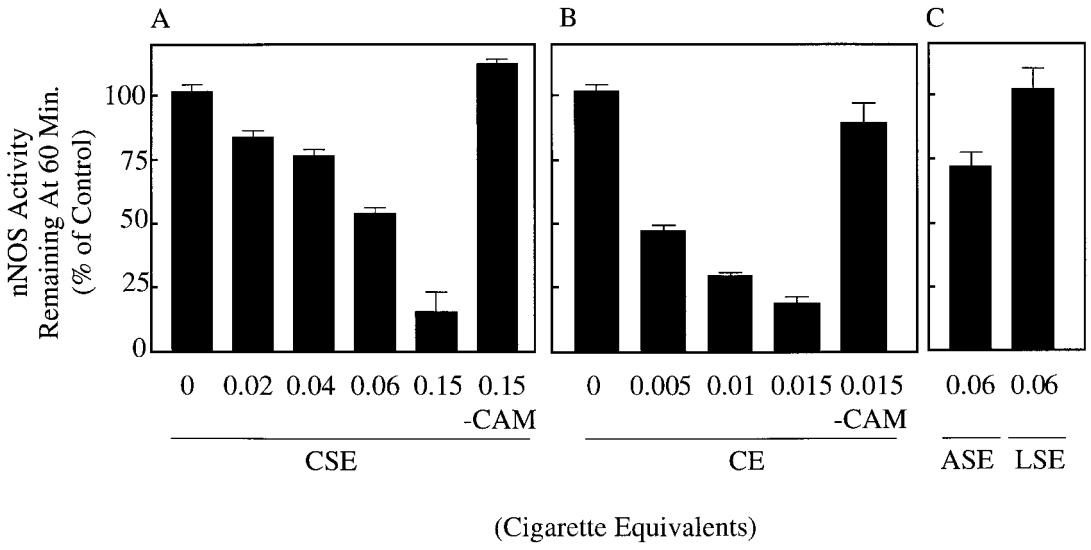


FIG. 2. *Concentration-dependent inactivation of nNOS by cigarette extracts.*

The amount of nNOS activity remaining after a 60-min incubation of the first reaction mixture with cigarette extracts at the indicated concentrations was determined. The activity is presented as a percentage of the nNOS activity from samples that have been incubated without extract for 60 min. A, treatment with aqueous extract made from the smoke of Camel cigarettes (CSE); B, treatment with aqueous extract made from Camel cigarettes (CE); C, treatment with aqueous extract made from the smoke of ASE or LSE. The values are the mean ± S.E. ($n$ = 3).

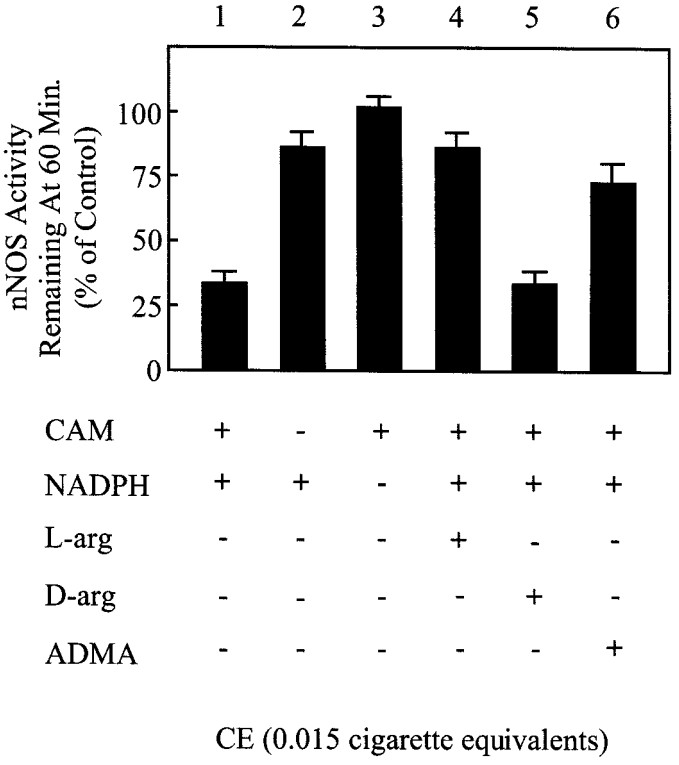


FIG. 3. *Effect of calmodulin, NADPH, arginine, and asymmetric dimethyl-L-arginine on the inactivation of nNOS caused by cigarette extract.*

The nNOS inactivation was determined as described under *Materials and Methods* except that where indicated, calmodulin or the NADPH-generating system were omitted from the first reaction mixture. As indicated, in some experiments 0.1 mM L-arginine, 1 mM D-arginine, and 100 $\mu$M ADMA were added in the first reaction mixture. The values are the mean ± S.E. ($n$ = 3).

TABLE 1

*Properties of the nNOS inactivator(s) present in CE.*

The CE was treated under various conditions, as described under *Materials and Methods*. The untreated CE (None) or the various CE preparations (0.015 cigarette equivalents) were added to the first reaction mixture, and the activity of nNOS was determined after a 60-min incubation. The values are the mean ± S.E. ($n$ = 3).

| Treatment of CE | nNOS Activity Remaining at 60 Min |
|---|---|
| | *% Control* |
| None | 28.2 ± 2.3 |
| Evaporation by SpeedVac apparatus | 29.0 ± 3.6 |
| Ultrafiltration with a 3,000-Da molecular mass cut-off membrane | |
| Retentate | 102.3 ± 2.0 |
| Filtrate | 24.7 ± 4.3 |
| Methylene chloride extraction | |
| Fraction | |
| 1 | 86.3 ± 6.0 |
| 2 | 102.0 ± 4.2 |
| 3 | 86.7 ± 5.5 |
| 4 | 34.0 ± 4.6 |
| Binding to cation exchange columns | |
| Flow-through | 92.3 ± 6.7 |
| Eluent of 2 M NaCl | 26.0 ± 4.6 |
| Binding to anion exchange columns | |
| Flow-through | 18.0 ± 5.0 |
| Binding to activated charcoal | |
| Supernatant after treatment | 102.3 ± 3.7 |

inactivation caused by the flow-through fraction. This was confirmed as the inactivator(s) was eluted off the column with 2 M NaCl. On the other hand, the inactivator(s) did not bind to an anion exchange resin and indicates that the compound(s) are either not anionic or only weakly anionic so as it could not displace the chloride ions on the resin. The small size, water solubility, and cationic nature suggested that the inactivator may be a small hydrophilic organic compound or perhaps a metal. To try to differentiate from these possibilities, 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 inactivation caused by the supernatant. Thus, the inactivator is likely an organic, small molecular mass, cationic compound(s). Heating of CE in boiling water for 5 min had no effect on the ability of the CE to inactivate nNOS.

Last, we tested research reference cigarettes obtained from the Tobacco and Health Research Institute, University of Kentucky (Lex-

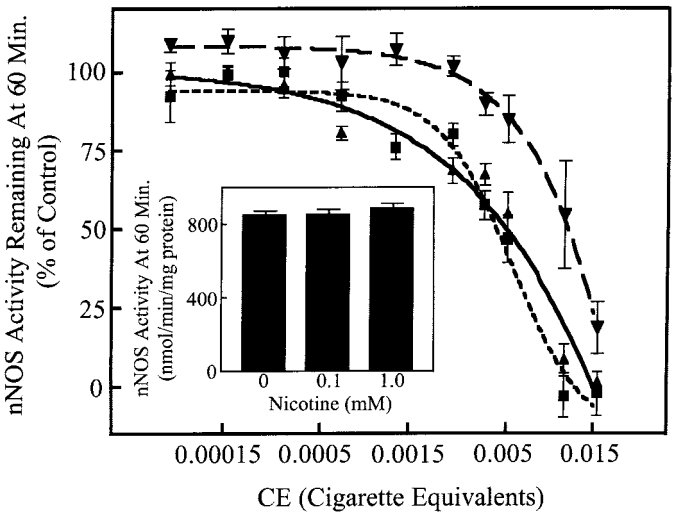


FIG. 4. *Effect of nicotine and extracts made from research grade cigarettes on nNOS.*

CE was made from research grade cigarettes 4A1 (▼, dashed line), 1R3 (▲, dotted line), and commercial Camel cigarettes (■, solid line). The effects of these extracts on nNOS are shown. The values are the mean ± S.E. ($n = 3$). Inset, nicotine at the indicated concentrations was added to the first reaction mixture and tested for effects on nNOS activity.

ington, KY). These cigarettes were produced as a reference standard for comparing data. As shown in Fig. 4, cigarette 4A1 (▼, dashed line) and cigarette 1R3 (▲, dotted line) caused a dose-dependent inactivation of nNOS with an $IC_{50}$ of 0.008 and 0.005 cigarette equivalents, respectively. The commercial grade Camel cigarettes (■, solid line) are shown for comparison with an $IC_{50}$ of 0.005. The 4A1 cigarettes are similar to the 1R3 cigarettes except that 4A1 has a much lower nicotine content. This difference, however, was not the cause of the greater potency of the 4A1 cigarette as nicotine, up to a concentration of 1.0 mM, did not inactivate nNOS (inset).

## Discussion

We have described the direct inhibition and inactivation of nNOS by cigarette constituents. The water-soluble extracts from cigarette and cigarette smoke contain compounds that cause a time-dependent inactivation of nNOS. Excess substrate or tetrahydrobiopterin does not readily reverse this inactivation. The time-dependent inactivation is more prominent in studies where the water-soluble extract from the unburned cigarette is used whereas the extract from the cigarette smoke contains compounds that appear to be competitive inhibitors of nNOS. Competitive inhibitors are known to mask the time-dependent inactivation process. The time-dependent inactivation occurs only in the presence of NADPH and calmodulin and suggests that a catalytically competent nNOS is needed for the inactivation event to occur. Moreover, the natural substrate protects nNOS from inactivation by cigarette constituents, suggesting that an active site-directed process is involved. Although the exact mechanism remains to be defined, these observations are consistent with a mechanism whereby some chemical(s) in the cigarette is metabolized by nNOS to a reactive intermediate that leads to alteration and inactivation of the enzyme. The reactive intermediate can potentially modify key amino acids of nNOS and/or a prosthetic group needed for nNOS function (Osawa and Pohl, 1989).

The mechanism of inactivation may be of importance in understanding why the nNOS protein is decreased in penile (Xie et al., 1997) and brain tissue (Hasan et al., 2001) after exposure of rats to smoke. The accelerated degradation of nNOS has been observed with several other metabolism-based NOS inhibitors, including $N^G$-methyl-L-arginine, $N^5$-(1-iminoethyl)-L-ornithine, and guanabenz (Nakatsuka et al., 1998; Noguchi et al., 2000). The loss of nNOS activity per se is not the signal for proteolytic removal since reversible inhibitors such as $N^G$-nitro-L-arginine and 7-nitroindazole did not enhance degradation of nNOS and may have actually stabilized the protein (Nakatsuka et al., 1998; Noguchi et al., 2000). There is precedence for metabolism-based or suicide inactivators to cause the enhanced proteolytic turnover of the affected enzyme. Especially pertinent are the examples described below from the liver microsomal cytochrome P450 enzymes, which are related to NOS (Alderton et al., 2001). It appears that structural changes and not the functional inactivation per se is the "trigger" for proteolysis of liver microsomal P450 cytochromes (Correia et al., 1987; Tierney et al., 1992). Moreover, the crosslinking of heme to protein plays a major role in the proteolytic recognition, whereas covalent alteration of the heme or the protein do not appear to be involved (Tierney et al., 1992; Korsmeyer et al., 1999; Wang et al., 1999). Indeed for nNOS one of the established targets of reactive intermediates is the heme prosthetic group, which is covalently altered to form heme adducts, including those crosslinked to the protein (Jianmongkol et al., 2000; Vuletich et al., 2002). For example, heme alteration and protein alteration occurs for $N^G$-methyl-L-arginine and $N^5$-(1-iminoethyl)-L-ornithine (Olken et al., 1994; Bryk and Wolff, 1999).

The nature of the constituent or constituents that are responsible for inactivating nNOS is still unknown. We have, however, ruled out nicotine, CO, and NO by our studies. The inactivator(s) appears to be a low molecular mass, nonvolatile, organic, cationic compound(s). This is not unexpected as the substrates for the enzyme are relatively low molecular mass polar compounds with cationic character. There are over 5,000 chemicals that have been characterized in tobacco smoke, including many with cationic character. It is also not clear if the same inactivator(s) is present in tobacco smoke as well as in the tobacco plant itself as burning may destroy and generate new inactivator(s). Indeed, the effects are more complex in the case of smoke as some competitive inhibitor(s) as well as time-dependent inactivator(s) are present. Interestingly, the smoke extract from the Accord cigarettes, which are heated but not burned, did not give a competitive inhibition of nNOS, suggesting that perhaps burning is needed to produce this type of nNOS inhibitor(s). We aim to delineate the exact chemical nature of the compounds that are responsible for these effects as unique inhibitors may be identified.

A dysfunctional eNOS is evident in smokers (Kharitonov et al., 1995; Higman et al., 1996; Heitzer et al., 2000; Barbera et al., 2001), and it is likely that eNOS is also directly inactivated by cigarette constituents. The results of Xie et al. (1997) indicate that tobacco smoke has a preferential effect on nNOS over eNOS in the penile tissue, although the mechanism of this selectivity is unknown. Perhaps there is a selective inactivation of nNOS over eNOS in this tissue. The further study of the mechanism of action of tobacco constituents is needed to better understand this selectivity and the complex actions of tobacco smoking on NO synthesis.

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