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Naphthoquinones and Bioactive Compounds from Tobacco as Modulators of Neuronal Nitric Oxide Synthase Activity

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Abstract

Studies were conducted with extracts of several varieties of tobacco in search of neuronal nitric oxide synthase (nNOS) inhibitors which may be of value in the treatment of stroke. Current therapies do not directly exploit modulation of nNOS activity due to poor selectivity of the currently available nNOS inhibitors. The properties of a potentially novel nNOS inhibitor(s) derived from tobacco extracts, and the concentration-dependent, modulatory effects of the tobacco-derived naphthoquinone compound, 2, 3, 6-trimethyl-1, 4-naphthoquinone (TMN), on nNOS activity were investigated, using 2-methyl-1, 4-naphthoquinone (menadione) as a control. Up to 31 μ M, both TMN and menadione stimulated nNOS-catalyzed *L*-citrulline production. However, at higher concentrations of TMN (62.5–500 μ M), the stimulation was lost in a concentration-dependent manner. With TMN, the loss of stimulation did not decrease beyond the control activity. With menadione (62.5–500 μ M), the loss of stimulation surpassed that of the control ($78 \pm 0.01\%$), indicating a complete inhibition of nNOS activity. This study suggests that potential nNOS inhibitors are present in tobacco, most of which remain to be identified.

Keywords

neuronal nitric oxide synthase; 2,3,6-trimethyl-1,4-naphthoquinone; menadione; quinones; tobacco; redox-cycling

INTRODUCTION

There are three main isoforms of nitric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) (Bredt and Snyder, 1990; Ignarro *et al.*, 1987; Forstermann *et al.*, 1991; Stuehr and Nathan, 1989; Hibbs *et al.*, 1991). Each NOS isoform is BH₄- and O₂-dependent and, utilizing NADPH as a co-substrate, NOS catalyzes the conversion of *L*-arginine to *L*-citrulline plus NO• (Kwon *et al.*, 1990).

Although all three NOS isoforms metabolize *L*-arginine to yield the same products, the function of the NO• that is produced varies widely depending on the tissue type and location

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(rev. by Ignarro, 1990). For example, NO• produced by eNOS modulates vascular dynamics (blood pressure and blood flow) *via* interactions with smooth muscle cells of the vascular endothelium (Ignarro *et al.*, 1988; Furchgott *et al.*, 1988; Pollock *et al.*, 1991), NO• produced by nNOS modulates chemical messaging within the central nervous system (Knowles *et al.*, 1989) while NO• produced in activated macrophages by iNOS is used for cellular defense (Hibbs *et al.*, 1988). The beneficial effects of NO• occur, for the most part, at low NO• concentrations (high nM to low μ M) and/ or in controlled cellular microenvironments. However, when excessive NO• is produced, tissue damage may occur. It has been suggested that both iNOS and nNOS may produce copious amounts of NO• during the course of many neurodegenerative disease states, spinal injury, as well as during and after stroke.

During stroke, the highly regulated production of NO• by nNOS (early phase) and iNOS (late phase) becomes dysregulated, leading to elevated local concentrations of not only NO•, but also NO•-derived reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Ischiropoulos *et al.*, 1995; Thom *et al.*, 1997). The elevated levels of ROS and RNS under stroke may increase the extent of damage resulting from the brain infarct (Nagafuji *et al.*, 1992; Huang *et al.*, 1994). Studies on rodent models of stroke suggest that prevention of NO• overproduction during and immediately after stroke using nNOS inhibitors, averts the accumulation of NO• to toxic levels and thus decreases infarct size (Chabrier *et al.*, 1999; Spinnewyn *et al.*, 1999; Ashwal *et al.*, 1994; Goyagi *et al.*, 2001; Zhang *et al.*, 1996; Zhao *et al.*, 2000). On the other hand, gene knockout studies in mice have suggested that production of NO• by eNOS, immediately after stroke, can be beneficial in limiting ischemic damage by helping to maintain tissue perfusion, and thus oxygenation within the area affected by the stroke (Huang *et al.*, 1996). Collectively, these observations suggest that targeted drug development, aimed at identifying selective inhibitors of nNOS and iNOS could lead to promising treatments for patients experiencing ischemic stroke (Dalkara *et al.*, 1994; Willmot *et al.*, 2005). It is clear that treatments for stroke, targeted at modulating the NO• pathway, will depend on the identification of NOS inhibitors which possess absolute specificity for the desired NOS isoform.

In addition to its role in modulating tissue damage in many neurodegenerative diseases and stroke, the NO• pathway is also actively involved in modulating neurotoxicity originating from environmental exposure to various chemicals including those present in both tobacco leaf as well as cigarette smoke. Tobacco smoke, which is composed of pyrolysis or pyrosynthesis products of leaf tobacco, possesses thousands of bioactive compounds (Stedman, 1968; Green and Rodgman, 1996). Recently, studies have focused on identifying nNOS inhibitors present in aqueous extracts of cigarette tobacco (Demady *et al.*, 2003) as well as in tobacco leaves (Lowe *et al.*, 2007).

Earlier studies reported that the aqueous extracts of cigarette tar contained a quinonesemiquinone-hydroquinone cycling system. The auto-oxidizable compounds present in this redox-cycling system of cigarette tar (Schmeltz *et al.*, 1977) may act as sources for the production of ROS, such as superoxides ($O_2^{\cdot-}$), via NAD(P)H- dependent mechanisms (Winston *et al.*, 1993). Since many redox-active compounds, such as quinones, possess the ability to interact with NAD(P)H-dependent, flavin-containing enzyme systems, we have focused on identifying compounds derived from tobacco leaves of various varieties that interact with nNOS to regulate its activity.

However, studies utilizing tobacco leaves as a targeted library for identifying possible treatments for Parkinson's disease have uncovered an inhibitor of (another flavoenzyme like NOS) monoamine oxidase, 2,3,6-trimethyl-1,4-naphthoquinone (TMN) (Khalil *et al.*, 2000). TMN which was isolated from burley tobacco leaves and characterized by the Castagnoli

group (Khalil *et al.*, 2000), produced inhibition of both human MAO-A and MAO-B. Also, *in vivo* studies with mice demonstrated that TMN was neuroprotective in the MPTP model of neurodegeneration (Castagnoli *et al.*, 2003). In order to understand if TMN can potentially modulate nNOS activity, we performed nNOS activity assays, using menadione as a positive standard.

The following studies describe: 1) the properties of potentially novel nNOS inhibitor(s) derived from hexane tobacco extracts of TI-1565, and 2) the concentration-dependent, modulatory effects of the tobacco-derived quinone compound, TMN on nNOS activity. These studies provide insight into the mechanistic events leading up to, and during, modulation of nNOS activity. Furthermore, these studies provide a foundation for planning and conducting future investigations into the interactions of bioactive compounds derived from tobacco with various enzyme systems.

MATERIALS AND METHODS

Chemicals

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *L*-arginine-HCl, CaCl₂·4H₂O, bovine brain calmodulin (CaM), (6*R*)-5,6,7,8-tetrahydro-*L*-biopterin (BH₄), reduced nicotinamide adenine dinucleotide phosphate (NADPH), *L*-citrulline, ethylenediaminetetraacetic acid (EDTA), menadione and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Dowex 50WX8 was purchased from Supelco (Bellefonte, PA). [¹⁴C]-*L*-arginine (313 mCi/mmol) was obtained from American Radiolabel Chemicals (St. Louis, MO). Hexane, formic acid, acetonitrile and Scinti-Safe Plus 50%™ scintillation cocktail were purchased from Fisher Scientific. All other chemicals were from common suppliers and were of the highest grade commercially available.

Enzymes

Recombinant nNOS was over-expressed in *E. coli* and purified according to established methodology (Roman *et al.*, 1995).

Tobacco

Several varieties of tobacco seeds, *Nicotiana tabacum* (TI-464, TI-1565), *Nicotiana sandarae* (TW-152), *Nicotiana raimondii* (TW-109), *Nicotiana noctiflora* (TW-88) and *Nicotiana nesophila* (TW-87), were generously provided by Verne A. Sisson, Oxford Tobacco Research Station, North Carolina State University. These varieties of tobacco were grown under identical conditions in a greenhouse. Leaves were collected at maturity (~ 85 days), washed with de-ionized, distilled water and dried at 30°C in a dehydrator under a stream of filtered ambient air. The dried different tobacco leaves were then milled to generate fine powders which were stored at room temperature in air-tight tubes for later extractions. Tobacco powders were sequentially extracted in a three-step process utilizing different solvents: hexane (non-polar; lipophilic), methanol (polar; amphiphilic) and water (polar). For routine extractions, 2.5 g of tobacco powder was placed in a beaker containing 100 ml of hexane and mechanically stirred overnight. The resulting suspension was vacuum-filtered using a buchner funnel (Whatman # 1 paper). The hexane extract was stored and the resulting solids were transferred to a beaker containing 100 ml of methanol. Again, the solids were rapidly stirred in methanol overnight, vacuum-filtered, and the resulting solids extracted with deionized, distilled water utilizing the same overnight procedure. Each individual liquid extract was stripped of organic solvent (hexane, methanol) by rotary evaporation and lyophilized. The aqueous extracts were frozen over dry ice/acetone and lyophilized. The weight of each individual extract powder/oil was recorded along with starting powder/oil weight. Reduced extracts were then stored in a dessicator at -20°C.

Isolation and Purification of TMN

Burley tobacco were obtained from Murty Pharmaceuticals Inc. (Lexington KY) and, TMN was isolated and purified by the laboratory of Dr. Neal Castagnoli, as described previously (Khalil *et al.*, 2000)

[¹⁴C]-L-arginine to [¹⁴C]-L-citrulline conversion assay

The conversion of [¹⁴C]-L-arginine to [¹⁴C]-L-citrulline was used to estimate NOS activity. Dose-response relationships were established by titrating aliquots of the reconstituted tobacco extracts into the assay incubations. nNOS activity was determined in 250 µl reactions essentially as described in Nishimura *et al.* (1999). nNOS reactions consisted of (final assay concentrations): L-arginine.HCl (20 µM), [¹⁴C] L- arginine (0.14 µCi), CaCl₂·2H₂O (200 µM), CaM (200 nM), BH₄ (5 µM), NADPH (100 µM) and nNOS (30nM) in 50 mM HEPES/KOH, pH 7.5. Lyophilized tobacco extracts were dissolved in water (aqueous extract), 10% DMSO in 50 mM HEPES (methanol extract) or DMSO (hexane extract). TMN or menadione were dissolved in 100 % DMSO. Depending on the experiment, either the tobacco extracts or TMN or menadione was added to the assay. Reactions were initiated by the addition of nNOS. Incubations were run for 5 mins at 23°C. Reactions were then quenched with an ice-cold stop solution consisting of 1 mM L-citrulline and 10 mM EDTA in 100 mM HEPES, pH 5.5. Dowex columns were prepared by converting the resin to the Na⁺ form by exposure to 5M NaOH for 20 mins followed by a water wash which was repeated 3-4 times or until the pH approached neutrality (~ pH 7.2). Two ml of resin was then used to prepare each column. Quenched reaction mixtures were placed on previously prepared Dowex columns and then washed twice with 1mL portions of deionized water to effect elution of [¹⁴C]-L-citrulline. The amount of ¹⁴C present as L-citrulline in the eluate was determined by liquid scintillation spectroscopy over a 2 min period. Control values from incubation containing all ingredients except enzyme and vehicle, were subtracted from all data points. Incubation containing all ingredients including nNOS except tobacco extract was considered as the basal control activity level. Data are expressed as the mean ± s.d. of duplicate determinations from three independent experiments and were analyzed using Sigma Plot 9.0 (Systat Software, Inc.).

NADPH Oxidation

The assay for nNOS-mediated NADPH oxidation consisted of 50 mM HEPES (pH 7.5), 100 µM L-arginine, 200 µM CaCl₂, 100 µM NADPH, 110 pmol CaM and +/- TMN and menadione (0-500µM). Reactions were started by adding 30 pmoles of nNOS to the appropriate sample tubes. Total reaction volume was 1 mL and reactions were monitored spectrophotometrically at 340nm for 60 seconds at 23 °C. Control incubations contained all ingredients except nNOS. The initial linear rate of NADPH oxidation was read against the “no nNOS” control. The rate of conversion of NADPH to NADP⁺ was calculated using $\epsilon = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$ (Miller *et al.*, 1997).

High Performance Liquid Chromatography (HPLC) Analysis of Tobacco Extracts

Chromatograms of the different tobacco extracts were obtained with a Shimadzu SCL-10AVP HPLC system equipped with a semi-preparative column (Beckman Coulter TM ODS 10 mm × 250 mm) at a flow rate of 2 ml/min. The eluate was monitored at 276 nm. Binary gradient elution was used to effect separation of the constituents present in the extracts from the different varieties of tobacco. The mobile phase solvents were acetonitrile (ACN) and 0.1% formic acid. The specific gradients used for separations were method A: ACN: 50%-100%, 30 min linear gradient for initial fractionation and method B: ACN: 50%-75%, 20 min linear gradient, followed by ACN: 75%-100%, 10 min linear gradient for sub-fractionation of TI-1565.

Mass Spectrometric Analyses

Mass analyses were conducted using mass spectrometry (MS) with electro-spray ionization (ESI) to probe HPLC fractions of the peaks of various tobacco extracts for predominant ions. Metal content was determined using ICP-OES. ICP-OES was used to analyze fractions of HPLC eluate from hexane extracts of TI-1565 for the presence of metal(s). MS and ICP-OES analyses were conducted in the Dept. of Chemistry facility at the University of Kentucky, Lexington, KY.

RESULTS AND DISCUSSION

Inhibition of *L*-citrulline Production by Tobacco Extracts

[¹⁴C]-*L*-arginine to [¹⁴C]-*L*-citrulline conversion assays were performed (see *Materials and Methods*) in the presence of different tobacco varieties that were extracted in hexane, methanol and water. Incubation containing all ingredients including nNOS except tobacco extract was considered as the basal control activity level. The vehicles of tobacco extracts were tested alone and had no significant effect on nNOS activity in the amounts used for experimentation. Results, showing the amount of inhibition of *L*-citrulline production by tobacco varieties, are grouped according to the particular solvent used for tobacco extraction:

Hexane—Hexane extracts of the five tobacco varieties designated TI-1565, TI-464, TW-109, TW-88 and TW-152 were potent nNOS inhibitors (60 - 86% inhibition of *L*-citrulline production) (Fig. 1A). Inhibition of *L*-citrulline production was observed even at the lowest concentration (125 µg/ml) tested. With the exception of TW-109, the hexane extracts of these tobacco varieties were by far more inhibitory of nNOS-catalyzed *L*-citrulline production than the corresponding methanol or aqueous extracts.

Methanol—Methanol extracts of TW-88 and TI-1565 showed a moderate inhibition (40 - 68% inhibition) of *L*-citrulline production (Fig. 1B). Furthermore, this inhibition occurred only at their higher concentrations indicating that these methanol extracts were less potent inhibitors of nNOS relative to the corresponding hexane extracts. Other tobacco varieties (TW-87, TW-109, TI-464, TW-152) could not be tested due to their poor solubility in the vehicle.

Water—Minimal inhibition of *L*-citrulline production (< 35% inhibition) was observed when nNOS was exposed to aqueous extracts of TI-1565, TW-88, TW-87 and TI-152 (Fig. 1C). Other tobacco varieties (TW-109 and TI-464) could not be tested due to their poor solubility in the vehicle. With the exception of the aqueous extract from TI-1565 (~50% inhibition of *L*-citrulline production), results indicated that, when compared to hexane or methanol extracts, the aqueous extracts were essentially not active as potent nNOS inhibitors in this system.

Additionally, inhibition of nNOS activity by tobacco extracts was estimated using cytochrome c reduction assay. In the cytochrome c reduction assay, cytochrome c couples well to nNOS reductase domain facilitating direct transfer of reducing equivalents from active nNOS to get reduced. Results showed that, with most tobacco varieties, cytochrome c reduction rate was very much lower for hexane extract when compared to methanol and aqueous extracts (data not shown). This indicated that hexane extracts of tobacco, in general, caused higher inhibition of nNOS activity when compared to methanol and aqueous extracts.

Collectively, these results suggest that inhibition of *L*-citrulline production by nNOS was dependent on the tobacco variety as well as the amount and apparent physico-chemical

properties of the compounds contained in various tobaccos. The rank-order potency of the tobacco extracts, in general, as inhibitors of *L*-citrulline production based on the solvent of extraction: hexane > methanol > water. From these observations, the most potent inhibitor(s) of *L*-citrulline production by nNOS are most likely non-polar and lipophilic in nature due to their extraction into hexane. Based on the higher nNOS inhibitory potency of hexane extracts relative to the corresponding methanol and aqueous extracts (Fig. 1), all subsequent efforts to isolate inhibitors of nNOS involved fractionation of the hexane extracts of the various tobaccos.

Qualitative HPLC Analyses of Hexane Extracts

Three different hexane extracts, TI-1565, TW-109 and TI-464 displaying high, moderate, or low nNOS inhibition, respectively, by [¹⁴C]-*L*-arginine to [¹⁴C]-*L*-citrulline conversion assay (Fig. 1A) were analyzed by reverse phase-HPLC. Individual lyophilized aliquots of each of these hexane extracts were separately dissolved at a concentration of 100 mg/ml in hexane. One hundred µl of each solution was then analyzed by HPLC with UV/Vis detection.

In comparing chromatograms from the three different varieties of tobacco, it was clear that each hexane extract possesses a unique and distinct chromatographic profile (data not shown). Obviously, differences in the amounts of the specific constituents and/or types of compounds present in the three different varieties of tobacco are responsible for the unique chromatographic profiles of the three various hexane extracts. It is tempting to speculate that differences in the detected (major) constituents, contained in each different variety of tobacco, are responsible for the differential nNOS inhibitory potencies of the extracts. However, differences in the potent inhibitor(s) present in low amounts in the three tobaccos should receive equal consideration.

Sub-Fractionation of the Hexane Extract of TI-1565

To investigate the nature of the compound(s) responsible for the nNOS inhibition, the hexane extract of the potent, inhibitory TI-1565 was sub-fractionated. HPLC eluates of TI-1565 were collected at intervals of 5 min following injection onto a reversed-phase column as described in 'Materials and Methods' (method A). The acetonitrile (ACN) from each pooled fraction was stripped by rotary evaporation and the aqueous volume further reduced (to 25 µl) by vacuum centrifugation (Jouan, Inc., VA) at 45°C. 5 µl of the resulting concentrated sample of every 5 min interval fraction was incorporated into the [¹⁴C]-*L*-arginine to [¹⁴C]-*L*-citrulline conversion assays and screened for activity of nNOS.

Results indicated that, with each successive 5 min sub-fraction that eluted between 0-15 min, there was an increase in percentage of inhibition of *L*-citrulline production catalyzed by nNOS (Fig. 2). This result further supports the idea that the potency of the individual nNOS inhibitor(s) is dependent on the relative lipophilicity of the responsible compound(s). Although these studies were initially focused on identifying inhibitors of nNOS activity, quite unexpectedly, each fraction eluting between 15- 30 min showed an increase in nNOS-catalyzed *L*-citrulline production indicating that there are both nNOS stimulating as well as inhibiting compound(s) in tobacco. Also, these results imply that a lipophilicity limit exists for compounds to illicit potent inhibition of nNOS.

During normal catalysis, nNOS is end-product inhibited, putatively up to 80%, due to the ability of the NO• to re-bind the heme iron from which it was produced (Ignarro *et al.*, 1990; Abu-Soud *et al.*, 1995; Abu-Soud *et al.*, 1996; Scheele *et al.*, 1999). Of those compounds that were identified as constituents of tobacco extract and/ or tobacco smoke, quinones or nitroarenes can interact with the nNOS flavins and lead to increased production of O₂⁻

(Miller *et al.*, 1997; Vásquez-Vivar *et al.*, 1997; Kumagai *et al.*, 1998). O_2^- acts as a trap for the enzymatically-generated $NO^\bullet$ to produce the potent oxidant, peroxynitrite ($ONOO^-$) at an almost diffusion-limited reaction rate ($6.7 \times 10^9 M^{-1} s^{-1}$; Huie and Padmaja, 1993; Scheme 1). This lowers the free $NO^\bullet$ concentration causing a decrease in the extent of $NO^\bullet$ -mediated auto-inhibition of nNOS (Griscavage *et al.*, 1995). This decreased level of nNOS auto-inhibition is then responsible for the concomitant corresponding increase in production of $NO^\bullet$ and *L*-citrulline. Compounds that increase product formation by NOS are, for the most part, redox-active (quinones / nitroarenes) or contain a redox-active metal (heme). A mechanism such as this may very well account for the activation of nNOS that was observed in our system following exposure of nNOS to various complex tobacco extracts.

In order to narrow the overall number of compounds in TI-1565 which could mediate nNOS inhibition, further sub-fractionation of the above-mentioned 5 min HPLC eluates displaying nNOS inhibition was performed. Separate 0.5 min HPLC eluate fractions of TI-1565 were collected and the fractions were concentrated and the aqueous volume was further reduced (to 30 μ l) as mentioned earlier. 5 μ l of the resulting concentrated sample of every 0.5 min interval fraction was incorporated into the [^{14}C]-*L*-arginine to [^{14}C]-*L*-citrulline conversion assays and screened for nNOS activity. Results showed that, the fraction eluting between 12.0-12.5 min produced the greatest degree of inhibition of *L*-citrulline production (~30 %, data not shown). Not surprisingly, no one single 0.5 min fraction (except 12.0-12.5 min fraction) produced the degree of nNOS inhibition that was observed with the initial 5 min fractions. We had expected to lose inhibitory activity at some point due to the ever-smaller fraction sizes of HPLC eluate which contained lower amounts of potential nNOS-inhibitory compounds. However, we did also observe many 0.5 min fractions of HPLC eluate which possessed the ability to stimulate nNOS activity (data not shown).

Mass Spectrometric Analyses and Sub-Fractionation of TI-1565

During HPLC analyses of hexane extract of TI-1565 (as mentioned earlier and in 'Materials and Methods'), six predominant peaks were observed and the corresponding eluates were collected for further study by ElectroSpray Ionization-Mass Spectrometry (ESI-MS). Results from the ESI-MS indicated that the eluate fraction of 12.0-12.5 min (corresponded to peak 5; data not shown) which produced maximal inhibition of *L*-citrulline production by nNOS, generated a predominant ion at an *m/z* value of 359. Eluate containing this ion was subjected to further fragmentation by tandem MS (MS/MS). However, interpretation of the resulting daughter ion profile was inconclusive.

Considering that a predominant mass ion peak was observed during MS analysis of the 12.0 - 12.5 min HPLC fraction (method A), and that the maximal nNOS inhibitory activity was contained within the 10-15 min fraction of HPLC eluate (method A), the time interval of 9-13 min was chosen for further sub-fractionation of HPLC. As a next step to further decrease the complexity of the fraction responsible for nNOS inhibition yet maintain an adequate concentration of putative inhibitor(s), fractions of HPLC eluate collected between 9-13 min from several runs were pooled and concentrated. Since the resulting 9-13 min pooled fraction of HPLC eluate still contained many compounds, the HPLC method was changed to method B as described in 'Materials and Methods' in an effort to further resolve peaks and thus increase purity upon isolation of potential nNOS inhibitory compounds. When method B was employed, the original 9-13 min fraction (method A) eluted between 21-29 min. Using method B, the 0.5 min fractions of HPLC eluate between 21-29 min were collected and pooled from 8 individual HPLC runs. The fractions were concentrated and the volume was reduced (to 20 μ l) as mentioned earlier. 5 μ l of resulting concentrated samples were incorporated into the [^{14}C]-*L*-arginine to [^{14}C]-*L*-citrulline conversion assays. Results showed significant nNOS inhibition (~60%) with 0.5 min fractions eluting between the 28-29 min (which corresponded to ~ 12-13 min eluate of HPLC method A). Due to the

nature of the isolation and concentration steps, recovery of this inhibitory constituent indicated that the nNOS inhibitor in this fraction from TI-1565 was non-volatile in nature. However, except 28-29 min fractions, there was no significant inhibition of nNOS activity (less than 20%) produced by any of the other 0.5 min fractions eluting between 21-29 mins. Despite our efforts to increase or maintain the concentration of putative inhibitor(s), the lack of inhibition was again most likely due to dilution and thus reduction in the concentrations of constituents present in each subsequent sub-fractionation. We speculate that the concentration of putative inhibitor(s) left in these sub-fractions was too low to produce significant inhibition of nNOS.

Interestingly, the major inhibitory fraction eluted between 28 - 29 min (corresponding to 12-12.5 min eluate of method A), on pooling over several runs and concentrated to a final volume of 20 μ l, appeared blue in color. Using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES), we probed the sample for metal content using copper, cobalt, nickel, chromium and vanadium. Results indicated the presence of trace amounts of copper in the concentrated sample. Copper levels were however, far from being stoichiometric amounts. When the [14 C]-*L*-arginine to [14 C]-*L*-citrulline conversion assay was performed in the presence of authentic copper sulfate or copper chloride (up to 10 mM each), there was no loss of *L*-citrulline production (data not shown) indicating that copper, by itself, or in these salt forms does not cause nNOS inhibition. However, the distinct color of the sample tempts us to speculate that the nNOS inhibitory compound could be organic in nature and possess unconjugated double bonds which produce the blue color. Quinones, for example, exhibit various colors depending on number of double bonds and/or oxidation state. Studies are needed to further characterize the physical or chemical properties of the nNOS inhibitory compound(s) in tobacco.

Differential Regulation of nNOS Activity by Naphthoquinones

In parallel with the investigations to identify nNOS inhibitors from tobacco extracts, studies were initiated using 2,3,6-trimethyl-1,4-naphthoquinone (TMN), a tobacco-derived flavoprotein inhibitor, and the structurally related 2-methyl-1,4-naphthoquinone (menadione) to evaluate their inhibitory effects on nNOS activity. TMN was isolated from burley tobacco leaves as described (Khalil *et al.*, 2000).

Concentration-response curves were generated for the TMN- and menadione- mediated regulation of *L*-citrulline production by nNOS, using [14 C]-*L*-arginine to [14 C]-*L*-citrulline conversion assay. The use of menadione as a standard or positive control, for redox-cycling allowed us to compare the concentration-dependent effects of TMN on nNOS activity. Assays without Menadione or TMN were used as a control activity to estimate the stimulation or loss of stimulation of *L*-citrulline production. Control values from incubation containing all ingredients except enzyme and vehicle, were subtracted from all data points.

Menadione—When lower concentrations of menadione (0 - 31 μ M) were titrated against nNOS in the [14 C]-*L*-arginine to [14 C]-*L*-citrulline conversion assay, the rate of *L*-citrulline production was progressively stimulated (Fig. 3A) and it reached a maximum stimulation (67 ± 0.01 % increase above 'no menadione' control) at 6.25 μ M concentration of menadione and plateaued. On the other hand, when higher concentrations of menadione (62.5 - 500 μ M) were titrated against nNOS, stimulation of *L*-citrulline production rate was lost in a concentration-dependent manner (Fig. 3B). This loss of stimulation continued well below 'no menadione' control activity (78 ± 0.01 % decrease from 'no menadione' control) up to 500 μ M concentration, which was the limit of solubility of menadione in our system.

TMN—When lower concentrations of TMN (0 - 31 μM) were titrated into the [^{14}C]-*L*-arginine to [^{14}C]-*L*-citrulline conversion assays, an increase in *L*-citrulline production rate ($\sim 40\%$ above 'no TMN' control) was observed beginning at 0.39 μM and persisted up to 31 μM (Fig. 3C). In contrast, when higher TMN concentrations (62.5 - 500 μM) were incorporated, stimulation of rate of *L*-citrulline production was gradually lost in a concentration-dependent manner ($27 \pm 0.04\%$ decrease from the maximum *L*-citrulline production rate at 62.5 μM), up to 500 μM which was also the limit of solubility of TMN in our system (Fig. 3D). Here, the loss of stimulation of *L*-citrulline production rate did not go below 'no TMN' control activity.

Both menadione and TMN, at lower concentrations stimulated *L*-citrulline production rate in a concentration-dependent manner and finally plateaued (Fig. 3A & Fig. 3C). Redox cycling agents and/or electrophiles can alter nNOS activity by different mechanisms. Quinones, in general, can influence nNOS activity in two ways: 1) by acting as single-electron acceptors and then autoxidizes to create a redox cycle and/or 2) by binding to protein thiol groups and causing covalent modification of the nNOS protein. Quinones can act as single-electron acceptors from flavoprotein reductases (Iyanagi and Mason, 1973; Iyanagi, 1990; Matsuda *et al.*, 2000) such as the nNOS reductases and cytochrome P450 oxidoreductase to yield a semiquinone radical. The semiquinone radical then transfers its one electron to O_2 via auto-oxidation to yield superoxide ($\text{O}_2^{\cdot -}$; Kappus & Sies, 1981). The resulting redox-cycle then results in unchecked O_2 reduction and concomitant production of $\text{O}_2^{\cdot -}$ at the expense of pyridine nucleotide (NADPH) oxidation. The increase in nNOS activity observed at various concentrations of TMN and menadione is most likely due to increased $\text{O}_2^{\cdot -}$ production from this futile redox cycle which traps the $\text{NO}^\bullet$ produced by nNOS and prevents, at least part, of the $\text{NO}^\bullet$ -mediated end-product inhibition (Scheme 1).

When higher concentrations (62.5 -500 μM) of TMN and menadione incorporated assays were compared, both compounds produced loss of stimulation of *L*-citrulline production rate in a concentration-dependent manner (Fig. 3B & Fig. 3D). These results suggest that, at higher concentrations of TMN and menadione, there may be insufficient electron flux remaining in nNOS to support $\text{NO}^\bullet$ production at the heme which also causes decreased *L*-citrulline production. The concentration-dependent decrease in *L*-citrulline production rate by nNOS is consistent with the concomitant increase in NADPH oxidation rate observed in the presence of both TMN and menadione (Fig. 4). At 125 μM , TMN began precipitating visibly. At higher concentrations of these compounds ($>125 \mu\text{M}$), the limit for electron transfer is reached, although different maximal rates occur for TMN and menadione (Fig. 4). Such inhibition was shown to increase in potency with increase in one-electron reduction potentials of those quinones (Kumagai *et al.*, 1998).

The possibility that quinones and semiquinones of TMN and menadione may covalently bind and/or oxidize thiol groups in the nNOS protein seems unlikely. This is because, TMN and menadione were present in great excess (up to 500 μM each), but only catalytic amounts of nNOS (7.5 pmoles) were added. Furthermore, the observed loss of stimulation of *L*-citrulline production in the presence of TMN and menadione is concentration-dependent and the covalent modification on nNOS due to quinones and semiquinones, would most likely not follow in a concentration-dependent manner.

The loss of stimulation of *L*-citrulline production rate (at 500 μM) by menadione continued well beyond control (no menadione) causing an outright inhibition ($78 \pm 0.01\%$ decrease from 'no menadione' control) of nNOS activity whereas the loss of stimulation of *L*-citrulline production rate (at 500 μM) by TMN did not go beyond control activity (no TMN) (Fig. 3B and Fig. 3D). This difference in their pattern of modulation of nNOS activity is most likely due to inherent redox-differences between TMN and menadione and/or due to

structural differences which increase or decrease quinone-enzyme interactions. Menadione acts as a true inhibitor of nNOS activity (Fig. 3B) whereas the addition of methyl groups in 3 and 6 positions of TMN (when compared to menadione) abolishes nNOS inhibitory activity (Fig. 3D). Thus, TMN at higher concentrations (62.5 - 500 μ M), causes only the loss of *L*-citrulline production rate and does not act as a true nNOS inhibitor at the concentrations tested (Fig. 3D). Collectively, these results indicate that TMN and menadione act as concentration-dependent modulators of nNOS.

The search for nNOS inactivator(s) in tobacco is an active field of study. Cigarette smoking has been shown to reduce exhaled NO• in humans suggesting that exposure to tobacco smoke alters the synthesis or disposition of NO• (Kharitonov *et al.*, 1995). Aqueous extracts of both cigarette smoke and non-burned cigarette tobacco were shown to cause the suicide inactivation of nNOS, apparently by interacting with the *L*-argininebinding site (Demady *et al.*, 2003). Additionally, a time- and metabolism-dependent inhibition of eNOS by extracts of non-burned cigarette tobacco has been documented (Lowe *et al.*, 2005). It has been proposed that (*S*)-(-)-nicotine, the principal pharmacologically active substance in tobacco, is not responsible for the lowered NOS activity in the brains of smokers (Vleeming *et al.*, 2002). At present, it is clear that many potential NOS inhibitors residing in both cigarette smoke and non-burned tobacco remain to be identified.

CONCLUSION

Experiments were performed in an attempt to identify properties of various constituent(s) in tobacco extracts that produce nNOS inhibition. The exact number and nature of tobacco constituent(s) responsible for inactivating nNOS remain unknown. Results from [¹⁴C]-*L*-arginine to [¹⁴C]-*L*-citrulline conversion assays that incorporated different varieties of tobacco extracts indicate that the nNOS inactivator(s) is non-polar, lipophilic and non-volatile in nature. Tobacco extracts can vary widely in their chemical composition depending on the variety of tobacco. Variation in content of the major tobacco constituents was evident from comparing HPLC profiles of the various inhibitory hexane extracts. We have shown that there are both activators as well as inactivators of nNOS present in non-burned tobacco. We speculate that there are countless other compounds possessing activity towards nNOS which remain to be identified. Future studies will attempt to identify the exact chemical nature of the compound(s) contained within the hexane extracts of TI-1565 responsible for producing nNOS inhibition. Studies in which tobacco-derived redox-active compounds such as TMN or menadione was individually incorporated into [¹⁴C]-*L*-arginine to [¹⁴C]-*L*-citrulline conversion assays indicate that single compounds can produce either stimulation or loss of stimulation of *L*-citrulline production by nNOS with effect being dependent on the concentration of the compound used as well as the compound's electrochemical characteristics. Furthermore, studies into the mechanism of action of these tobacco constituents will be required in order to ascertain the isoform-selectivity of nNOS inhibition.

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ABBREVIATIONS

nNOS	neuronal nitric oxide synthase
HPLC	high performance liquid chromatography
NO•	nitric oxide
O₂^{•-}	superoxide anion radical
TMN	2,3,6-trimethyl-1,4-naphthoquinone
ROS	reactive oxygen species
RNS	reactive nitrogen species
MS	mass spectrometry
ICP-OES	inductively-coupled-plasma optical emission spectrometry
ACN	acetonitrile

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