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Regulation of LPS-induced Nitric Oxide Synthase Activity by Cigarette Smoke in Mouse Brain

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ABSTRACT. Nitric oxide (nitrogen monoxide, NO) plays important physiological roles, but excessive generation can be toxic. NO is present in cigarette smoke at up to 1,000 ppm, and probably represents one of the greatest exogenous sources of NO to which humans are exposed. We investigated whether cigarette smoking reduces the production of endogenous NO and whether it influences the action of lipopolysaccharide (LPS) to induce nitric oxide synthase activity in mouse brain. Mice (C57BL6/J) were exposed to cigarette smoke for 8 weeks. LPS was injected intraperitoneally in single or combination with the exposure to cigarette smoke. Six hours after the injection of LPS, mice were sacrificed and sera and brains were collected. Serum concentrations of nitrate and nitrite were not changed by 4-week smoke exposure, but were significantly increased by 6 and 8 weeks of smoke exposure. Interestingly, cigarette smoke reduced the elevation in serum nitrate and nitrite concentrations produced by LPS after 4-week smoking exposure. NO synthase (NOS) activity in brain was upregulated by LPS-administration. However, cigarette smoke exposure remarkably and consistently decreased the LPS-induced activity in mouse brain. This result suggests that cigarette smoking may affect against overproduction of the endogenous NO by LPS through the inhibition of NOS activity induced by LPS in brain or by modulation of the LPS action for the induction of NOS activity. We also suggest the possibility that the exogenous NO evolved in cigarette smoke enables feedback inhibition of NOS activity or other possibility that it attenuates the toxicity of endotoxin LPS *in vivo* by unknown mechanisms, which should be further studied.

Keywords: Nitric oxide synthase, Lipopolysaccharide, Cigarette smoking, Nitrite, Nitrate.

INTRODUCTION

Nitric oxide (NO), a reactive free radical gas, has been widely recognized as a key mediator of metabolic homeostatic processes, host defense mechanisms, and oxidant tissue injury (Nathan and Xie, 1994). NO is reported to participate in the physiology or pathophysiology of mammalian systems (Nathan, 1992). In the brain and peripheral nervous system, NO displays many properties of a neurotransmitter. However, NO is also implicated in neurotoxicity associated with stroke and neurodegenerative diseases (Hope *et al.*, 1991; Nowicki *et al.*, 1991; Bredt and Snyder, 1992), neural regulation of smooth muscle including peristalsis (Desai *et al.*, 1991; Bult *et al.*, 1990), and penile erection (Burnett *et al.*, 1992). NO is also responsible for endothelial-derived relaxing factor activity regulating blood pressure (Moncada *et al.*, 1991; Ignarro, 1989). In macrophages NO mediates tumoricidal and bactericidal actions, because inhibitors of NO synthase (NOS) block these effects (Nathan and Hibbs, 1991).

NO is produced from L-arginine by oxidation of the terminal guanidine-nitrogen arginine by NOS, of which there are three distinct isoforms (Nathan and Xie, 1994). Two of these are dependent for their activity on intracellular calcium levels and were first demonstrated to be constitutively expressed in nervous tissue (NOS I, ncNOS) and endothelium (NOS III, ecNOS). The other

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isoform (NOS II, iNOS) is independent of intracellular calcium and its activity is controlled principally at the levels of transcription and translation. iNOS is induced in endothelium, vascular smooth muscle, and activated neutrophils and macrophages by pro-inflammatory cytokines and lipopolysaccharide (LPS).

Endotoxin LPS, bacterial membrane component, induces the production of host inflammatory mediators such as tumor necrosis factor α, interferon γ (INF-γ), and interleukin 1β, which in turn cause an increase in the expression of iNOS. The large amount of NO produced by iNOS has been hypothesized to contribute to the hypotension of endotoxic shock (Leblanc *et al.*, 1992) and there is thus considerable interest in strategies for inhibiting NO *in vivo*.

Besides the endogenous production, NO can also be found in high amounts in cigarette smoke (Norman and Keith, 1965). The gaseous phase of cigarette smoke has also been shown to induce an increase in the activity of guanylate cyclase, an enzyme which can be activated by NO, suggesting that NO is responsible for the observed effects of cigarette smoke (Arnold *et al.*, 1977; Gruetter *et al.*, 1980). It was reported that endogenous NO production was reduced in smokers (Persson and Gustafsson, 1993; Persson *et al.*, 1994; Hill *et al.*, 1995). Cigarette smoke extract was shown to inhibit the enzyme activity of NOS in bronchial epithelial cells (Robbins *et al.*, 1993). Although cigarette smoke itself contains high amounts of NO, no data are available whether the NO content of cigarette smoke may be absorbed to counteract the NOS activity in mouse brain tissue so far. Thus, in this study we investigated whether cigarette smoke affects the production of endogenous NO and whether it regulates NOS activity induced by LPS in mouse brain.

MATERIALS AND METHODS

Reagents

L-[2,3,4,5-^{3}H] Arginine hydrochloride was from Amersham Life Science Limited (UK). cNOS and iNOS antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Kentucky 1R4F reference cigarettes (0.8 mg nicotine and 11.5 mg tar per cigarette) were from the Tobacco and Health Research Institute, Lexington, KY (Diana and Vaught, 1990). Bacterial LPS (*E. coli* serotype 026:B6), tetrahydrobiopterin, calmodulin, and all the other general reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Animals

Male C57BL6/J mice (6 weeks of age) were used in the study. The mice were housed at the animal house at KT&G Research Center (Korea) under a 12 h dark/light cycle and were allowed food and water *ad libitum*. In the morning of the experiment the mice were exposed to cigarette smoke generated from 15 Kentucky 1R4F research cigarettes for 10 min a day and 5 days per week by using the automatic smoking machine (Heiner Borgwaldt, Germany).

Two *in vivo* treatments were conducted for these studies: smoking- and smoking plus LPS-injection. The animals were sacrificed with 6 h after single i.p. administration of physiological saline or LPS at 25 mg/kg and blood was obtained by cardiac puncture for analyses of NO_2^- and NO_3^-. The brain was also removed, dissected into cerebral cortex, hippocampus, hypothalamus, striatum, and cerebellum, and used for the analyses of NOS activity and immunodetections cNOS or iNOS.

Measurement of NO_2^- and NO_3^- concentration

Nitrite plus nitrate (NOx) in mouse serum samples was measured spectrophotometrically as described (Sherman *et al.*, 1993) using the Griess reagent. To do this, serum was deproteinized before analysis as follows. Ten microliters of 15% (w/v) trichloroacetic acid was added to a 60-μl serum. Treated serum was mixed by vortexing every 5 min, allowed to react for 30 min at room temperature, and then centrifuged at 12,000 x g for 15 min. Sixty microliters of supernatant were combined with 10 μl of 50 mM Hepes buffer (pH 7.5) and 5 μl of 0.75 M NaOH for neutralization. Nitrate was enzymatically reduced to nitrite with nitrate reductase, and nitrite was incubated with the same volumes of 1% (w/v) sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine for 10 min at room temperature and then determined by reading the absorbance at 540 nm. The $NaNO_2$ was used as a standard.

Nitric oxide synthase activity

NOS activity was determined as L-[^{3}H]citrulline formation from L-[^{3}H]arginine as described (Salter *et al.*, 1991). In these experiments, 50 μl of sample, corresponding to $\approx$ 100 μg of total cellular protein, was incubated at 37°C for 30 min in the presence, unless otherwise indicated, of 50 mM Tris-HCl buffer, pH 7.8, containing 0.5 μCi L-[^{3}H]arginine (305 mCi/mmol; Amersham Corp.; 1 Ci = 37 GBq), 0.45 mM $CaCl_2$, 10 mg calmodulin, 2 mM NADPH, 5 μM H_4biopterin, 5 μM FMN, 5 μM FAD, and 500vM Arginine in a total volume of 100 μl. Blank values were determined in the absence of enzyme. The reaction was terminated by the addition of 2 ml of ice-cold 20 mM Hepes (pH 5.5) containing 2 mM EDTA, and the total volume was applied to 1-

ml column of a Dowex AG 50W-X8 (Na^+ form) preequilibrated with the same buffer. After elution with further 2-ml of dH_2O, the concentration of L-[^{3}H] citrulline recovered in the effluent was determined by liquid scintillation counting.

Western blot analysis

Immunoblot analysis was done on lysates of brain homogenates by conventional procedures using mouse monoclonal antibodies to human nNOS and mouse iNOS (Transduction Laboratories, Lexington, KY) and an alkaline phosphatase-conjugated secondary antibody (Protoblot II® AP System, Promega) as follows: Brain homogenates were boiled for 4 min with gel loading buffer (50 mM Tris/10% SDS/10% glycerol/10% 2-mercaptoethanol/2 mg of bromophenol blue per ml) in a ratio of 1 : 4 and centrifuged at 10,000 x g for 10 min. Thirty mg of protein for each sample were separated on SDS/7.5% polyacrylamide minigels (Bio-Rad) using the Laemmli buffer system and transferred to nitrocellulose membranes. Nonspecific protein binding sites were blocked with 1% (w/v) blot qualified BSA in 20 mM Tris-Cl, pH 7.5, containing 150 mM NaCl and 0.05% (v/v) Tween 20, and the samples were incubated with either iNOS or nNOS antibodies (1 : 2,000). Bands were detected with an alkaline phosphatase-conjugated anti-mouse IgG and visualized with Western blue stabilized substrate, nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), for alkaline phosphatase. Prestained high molecular protein markers (Bio-Rad) were used for molecular size determinations. Protein concentrations of the supernatants were determined according to the method of Lowry *et al.* (1951).

Changes in the amount of ncNOS induced by cigarette smoke were determined with a LKB ultrascan laser densitometer. The band intensities were converted to electrophoretic peaks for analysis. The relative activity was calculated from the integration of peak areas.

RESULTS

Effects of cigarette smoke exposure on the serum level of nitrate and nitrite in LPS-induced mice

Fig. 1 shows the effect of cigarette smoke on the serum level of nitrate and nitrite in LPS-induced mouse. LPS significantly induced almost 15-fold increase of the NOx contents compared with cigarette smoke itself. Cigarette smoke itself gave no influence in the NOx contents up to 4-week exposure, but NOx contents were increased in the following consecutive exposure to 6 and 8 weeks. But these increases were negligible compared with the endogenous increase by LPS. This result indicated that NO in cigarette smoke, although inhaled at a high concentration, was quantitatively insufficient to affect the serum level of nitrate and nitrite in the mouse. Interestingly, cigarette smoke exposure reduced the induction of NO contents by LPS after 4-week smoking exposure, suggesting that the overproduction of endogenous NO by LPS is suppressed by cigarette smoke.

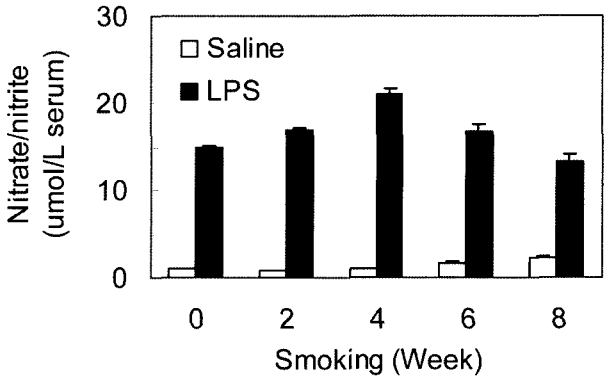

Fig. 1. *In vivo* changes of nitrate and nitrite levels in mouse serum by cigarette smoking and LPS treatment. Groups of mice were exposed to cigarette smoke during the periods indicated, and LPS was administered as described in Materials and Methods. Bars represent means ± SD for five mice.

Effects of cigarette smoke on the NOS activity in LPS-induced mouse brain

To understand the mechanism of inhibition of LPS-induced NO production in mouse brain by cigarette smoke, we examined the effect of cigarette smoke exposure on the formation of L-citrulline from L-argin-

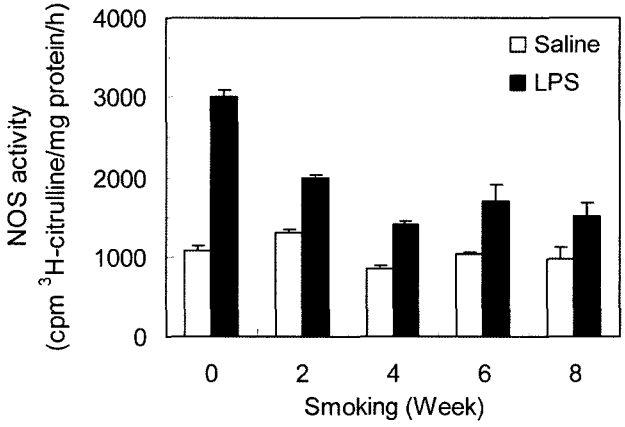

Fig. 2. Effect of cigarette smoking or LPS on NOS activity in mouse brain. Groups of mice were exposed to cigarette smoke during the periods indicated, and LPS was administered as described in Materials and Methods. Bars represent means ± SD for five mice.

ine, the reaction which is catalyzed by NOS in brain homogenates. Fig. 2 shows the effect of cigarette smoke exposure on the NOS activity in LPS-induced mouse brain. Cigarette smoke exposure itself up to 8 weeks did not give significant change of NOS activity in mouse brain. LPS administration to the non-exposed mouse to cigarette smoke induced the NOS activity in brain by 3-fold compared with vehicle-treated mouse brain. However, the LPS-administration to cigarette smoke-exposed mouse through the whole periods of exposure remarkably and consistently decreased the induction of NOS activity in mouse brain. This result suggests that cigarette smoke may inhibit the enzyme NOS activity in brain or modulate the action of LPS for the induction of NOS activity.

Effects of cigarette smoke exposure on the induction of NOS activity in mouse brain

Fig. 3 shows Western blot analysis of the expression of constitutive and inducible NOS induced by LPS in five regions of mouse brain. It showed that ncNOS in all the 5 brain regions tested was identified at 155 kDa position irrespective of LPS treatment (Fig. 3A), but iNOS, in spite of LPS administration, was not detected in any brain region (Fig. 3B). It has been reported that glial cells and astrocytes possess the inducible, Ca^{2+}-independent form of NOS (Galea *et al.*, 1992, 1994; Zielasek *et al.*, 1992), but it was not identified in brain tissue so far. iNOS antibody prepared by Transduction Laboratories (Lexington, KY, USA) also recognized a

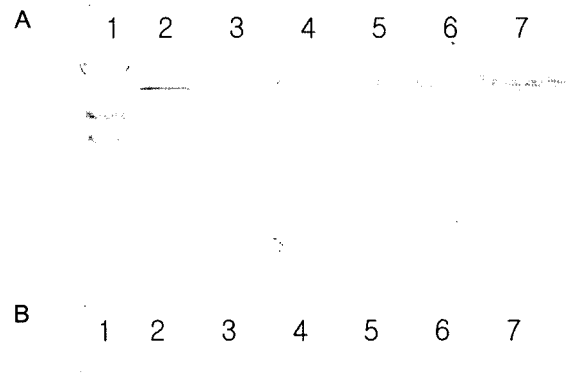

Fig. 3. Western blot analysis of the expressions of brain type NOS (A) and iNOS (B) induced by LPS in mouse brain. Western blot analysis was performed on samples transferred to nitrocellulose by an electroblot procedure, using mouse anti-NO synthase and alkaline phosphatase-conjugated anti-mouse IgG. Lanes: 1, M.W size markers (kDa); 2, Positive controls (Rat pituitary lysate for A; Mouse macrophage lysate for B); 3, Striatum; 4, Hippocampus; 5, Hypothalamus; 6, Cortex; 7, Cerebellum.

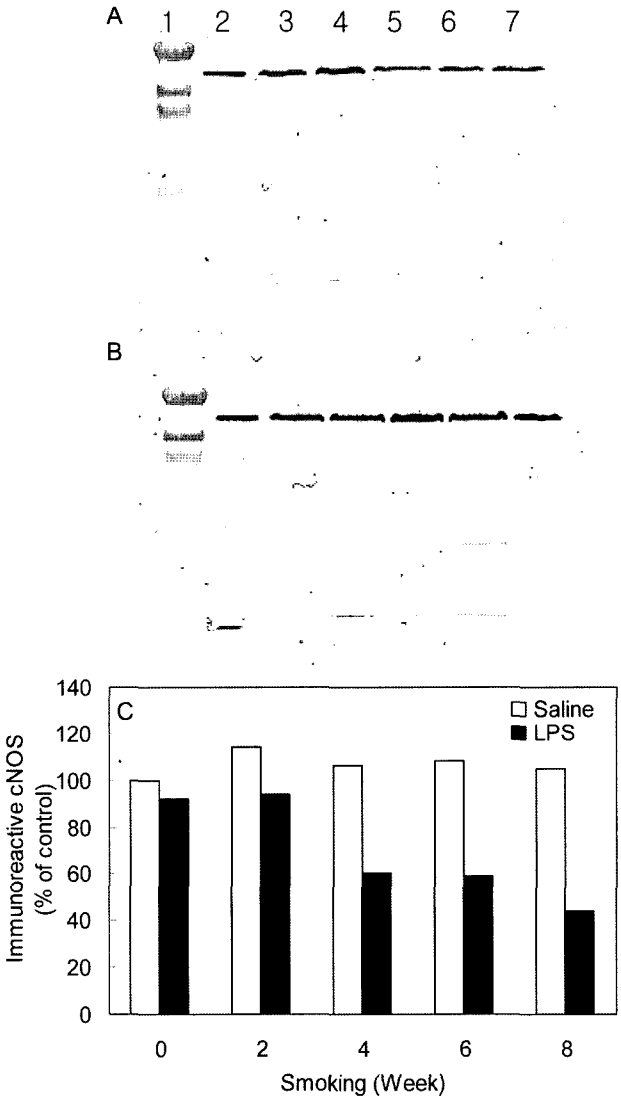

Fig. 4. Western blot analysis of cNOS protein expression induced by LPS or cigarette smoking in mouse striatum. Western blot analysis was performed on samples transferred to nitrocellulose by an electroblot procedure, using mouse anti-NO synthase and alkaline phosphatase-conjugated anti-mouse IgG. A. Group exposed to cigarette smoke; B. LPS administered and exposed to cigarette smoke group; C. Changes in the relative amount of cNOS. The relative amount was quantified by densitometry and expressed as optical density ratio (%). Lanes in A and B: 1, M.W size markers (kDa); 2, Positive controls (Rat pituitary lysate); 3, No smoking; 4, 2-week; 5, 4-week; 6, 6-week; 7, 8-week smoking.

protein band of 130 kDa in IFN and LPS-treated mouse macrophages but not in any region of brain tissue. Fig. 4A shows Western blot analysis for the expression test of ncNOS protein in striatum by cigarette smoking for maximum 8 weeks. It showed that almost the same density of ncNOS band was detected at 155 kDa position in the respective smoking periods, suggesting that cigarette smoke itself did not give any induction of ncNOS enzyme activity. Fig. 4B is the result of Western blot analysis for testing of the effect of cigarette smoke exposure on LPS-induced ncNOS activity in striatum, which showed in Fig. 4C that the density of ncNOS band at 155 kDa position was decreased as increasing the period of cigarette smoke exposure.

DISCUSSION

Since NO synthesis is important in many pathological conditions, there is a considerable interest in ways of inhibiting its synthesis *in vivo*. In the present study, we showed that cigarette smoke *in vivo* suppressed NO synthesis induced by a single dose of LPS as assessed by serum nitrate and nitrite levels, the enzyme activity, and immunoblot analysis in brain. Interestingly, cigarette smoking itself up to 8-consecutive weeks did not give any effect of NOS activity in mouse brain but remarkably attenuated the activity induced by LPS by inhibiting the expression of NOS protein.

Wennmalm *et al.* (1993) had studied the metabolism and kinetics of inhaled NO. They observed that the inhaled NO is metabolized by uptake into red blood cells with subsequent conversion to nitrate and MetHb. Nitrate then enters the plasma to be excreted via kidneys (Wennmalm *et al.*, 1993). Increased serum levels of nitrate in human were observed during inhalation of NO as well as in conditions characterized by enhanced activity of either the cNOS or iNOS (Green *et al.*, 1982; Jungersten *et al.*, 1993). This indicates that serum nitrate can be applied as measures of inhaled or endogenous NO activity *in vivo*.

Cigarette smoke yields as much as 400~1,000 ppm NO/40 ml puff (Norman and Keith, 1965). In our study, cigarette smoke attenuated LPS-induced increases in serum levels of NO and *in vivo* production of NO by NOS enzyme in brain. However, in our previous study, we failed to obtain the results that LPS induced the expression of mRNA for iNOS in brain. Undetectability in the induction of iNOS mRNA or enzyme iNOS by LPS in brain tissue made us difficult to get the conclusion that cigarette smoke may probably act at a transcriptional or translational level to inhibit the activity of NOS in brain, which remains for further study. However, our results suggest that majority of the exogenous NO produced by cigarette smoke is excreted out of the body with no influence on the enzyme NOS. Our results also suggest that the exogenous NO does not upregulate the NOS activity, rather downregulates the overproduction of endogenous NO by LPS. Therefore, from our result of undetectability in the induction of iNOS mRNA by LPS administration in brain tissue, it is predictable that the increase of NOS activity is thought to be enhanced formation or activity of cNOS, not to be induction of iNOS, by facilitating some cofactors, i.e., tetrahydrobiopterin, to the cNOS for the increase of the enzyme activity. Actually, there are some reports that LPS can increase the cNOS activity by increasing the accessibility of cofactors to cNOS protein (Lee *et al.*, 1995; Hattori *et al.*, 1997).

Recently it has reported that NO itself may have a feedback inhibitory effect on NOS activity in a variety of cell types (Assreuy *et al.*, 1993; Buga *et al.*, 1993; Rengasamy and Johns, 1994). There are some reports that cigarette smoking reduces the production of endogenous NO by inhibiting the enzyme NOS activity (Kharitonov *et al.*, 1995; Hill *et al.*, 1995; Schilling *et al.*, 1994; Robbins *et al.*, 1993). In the present study, brain type NOS activity upregulated by endotoxin LPS was attenuated in the presence of exogenous NO supplied from cigarette smoke, supporting the feedback inhibitory effect.

Although normally undetectable in brain tissue, iNOS mRNA has been observed in CNS tissues of animals under experimental pathologic conditions (Koprowski *et al.*, 1993). Our result that cigarette smoking reduces NO production by LPS administration may also reflect an inhibitory effect of cigarette smoke constituents on iNOS in CNS tissue. The effect of cigarette smoking may attenuate the property of LPS that induces the production of host inflammatory mediators or contributes to LPS-induced hypotension and mortality. Thus, the inhibitory effect of cigarette smoke on LPS-induced NOS activity may also assume great pathophysiological importance in neurodegenerative disease such as stroke, Alzheimer's disease, and Parkinson's disease. Therefore, our result, i.e., the inhibitory effect of cigarette smoke on LPS-induced NSO activity, may support the opinion of Wright *et al.* (1992) that selective inhibition of iNOS may prevent the early hypotension associated with bacterial sepsis and septic shock. In the future, it will be important to identify the constituents of cigarette smoke that are responsible for this inhibitory effect.

We believe that NO, present in high concentrations in the gas phase of fresh cigarette smoke, is the best candidate for contributing to the attenuation of LPS-induced NO generation. However, cigarette smoke is an extremely

complex mixture of particulate and gaseous materials. We cannot rule out the possibility of another substance, such as carbon monoxide (CO), to attenuate the effect. The concentration of CO in cigarette smoke is approximately 4% (Borkhoven and Niessen, 1961), which might inhibit NOS activity, because NOS has a heme structure related to cytochrome P450 and is potently inhibited by CO (White and Marletta, 1992). CO also shares some of the chemical and biological properties of NO. It is produced endogenously by various cell types as a byproduct of heme-oxygenase (Maines, 1988), and is being considered as an endogenous biological messenger in the brain (Verma *et al.*, 1993).

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