BIOMEDICAL AND ENVIRONMENTAL SCIENCES 16, 256-266 (2003)

Injury of Mouse Brain Mitochondria Induced by Cigarette Smoke Extract and Effect of Vitamin C on It *in vitro*

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Objective To investigate the toxicity of cigarette smoke extract (CSE) and nicotine on mouse brain mitochondria as well as the protective effect of vitamin C *in vitro*. **Method** Mouse brain mitochondria *in vitro* was incubated with CSE or nicotine in the absence or presence of vitamin C for 60 minutes, and the changes of mitochondrial function and structure were measured. **Results** CSE inhibited mitochondrial ATPase and cytochrome C oxidase activities in a dose-dependent manner. However, no significant changes in the peroxidation indices were observed when mitochondrial respiratory enzymes activity was inhibited, and protection of mitochondria from CSE-induced injury by vitamin C was not displayed *in vitro*. The effect of CSE on mouse brain mitochondria swelling response to calcium stimulation was dependent on calcium concentrations. CSE inhibited swelling of mitochondria at 6.5 μmol/L Ca^{2+}, but promoted swelling response at 250 μmol/L Ca^{2+}. Nicotine, the major component of cigarette smoke, showed no significant damage in mouse brain mitochondria *in vitro*. The CSE treatment induced mitochondrial inner membrane damage and vacuolization of the matrix, whereas the outer mitochondrial membrane appeared to be preserved. **Conclusion** The toxic effect of CSE on brain mitochondria may be due to its direct action on enzymatic activity rather than through oxygen free radical injury. Nicotine is not the responsible component for the toxicity of CSE to brain mitochondria.

Key words: Cigarette smoke extract; Nicotine; Vitamin C; Mitochondrial function; Mitochondrial structure

INTRODUCTION

Cigarette smoking remains the primary cause of preventable death and morbidity in many countries. The perinatal exposure to environmental cigarette smoke may develop toxic effects on brain development and incur events like sudden infant death syndrome[1]. Cigarette smoking may exert lasting adverse effects on brain structure and function as well as child's physical and cognitive growth[2]. An increased risk of Alzheimer's disease in smokers was reported in a prospective study[3]. The mitochondria is the key site for oxidative phosphorylation and ATP production in cells. It was reported that the internal membranes of the mitochondria incubated with cigarette smoke residue were degraded with time, correlating well with loss of ciliary activity and cell death. Therefore, it may well be suggested that mitochondria should be one of the principle damage sites of cigarette smoke[4].

It is well known that cigarette smoking increases the risk of coronary heart disease and contributes significantly to mortality of other cardiovascular diseases. And smoke-induced

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0895-3988/2003
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heart mitochondrial respiratory inhibition has been observed[5], implicating that the brain mitochondria may also be one of the targets predisposed to cigarette smoking damage.

Our study was to investigate the toxic effect of cigarette smoke extract (CSE) and nicotine on mouse brain mitochondrial function. Furthermore, in view of free radical-induced oxidative damage has been considered as an important mechanism responsible for the toxicity of cigarette smoking[6], we also studied the possible association of CSE-induced mitochondrial toxicity with an increase of oxidative damage and the protective effect of vitamin C on mitochondrial injury.

MATERIALS AND METHODS

Animals

Kunming strain mice obtained from the Animal Center, Chinese Academy of Medical Sciences were housed under standard laboratory conditions with free access to food and water. At the end of each experiment, equal number of female and male mice weighing 18-22 g were killed by decapitation. The skull of each mouse was rapidly opened, and the cerebral hemispheres were rapidly removed into ice-cold medium.

Materials

Nicotine ditartrate dihydrate, vitamins C and cytochrome C were purchased from Sigma Chemical Co. Ficoll 400 and Sephadex G-25 were obtained from Pharmacia, Ltd., Uppsala, Sweden. The other chemicals were of A.R. grade. DPH (1,6-diphenyl-1,3,5-hexatriene) stock solution (1 mg/mL) in absolute ethanol was prepared and stored in the dark at -20℃.

Preparation of Cigarette Smoking Extracts (CSE)

CSE was prepared by a modified method similar to that described by Su[7]. Smoke from one Marlboro cigarette (Philip Morris Products INC. Richmond, VA U.S.A.) was bubbled through 30 mL HEPES buffer, pH 7.4 by the apparatus shown in Fig. 1. And the smoke should be kept in the dark. The resulting solution, or primary CSE solution, was filtered through a 0.22 μm Millipore filter to remove particulates and bacteria. In each experiment freshly prepared CSE was used within 5 minutes after preparation and mixed gently.

Preparation of Mouse Brain Mitochondria

Freshly isolated mitochondrion were used in all the experiments. Mitochondrion from mouse brain were prepared by using the procedure of Przedborski[8], except that an isolation medium containing (in mmol/L) 210 manitol, 70 sucrose, 10 HEPES, 1 EDTA, 0.1 % (w/v) bovine serum albumin, pH 7.4 was used. All steps were performed at 4℃. After centrifugation, the final pellet was suspended in 280 mmol/L sucrose and 10 mmol/L HEPES, pH 7.4 to give a concentration of 15-20 mg/mL protein determined by Lowry method[9].

Determination of ATPase Activity

ATPase activity in three frozen-thawed mitochondrion was determined by the method described by Kielley[10]. The mouse brain mitochondrion were exposed to 10^{-6} mol/L nicotine or CSE at the concentrations of 0, 4, 8, 16, and 32 μL primary CSE solution/ml in the absence or presence of vitamin C for 60 minutes at 37℃, respectively. The reaction

buffer contained (in mmol/L) 0.25 mL of 0.20 histine buffer, 5 $MgCl_2$, 5 ATP, and 0.1 mg/mL mitochondrial protein. The tubes were incubated at 28℃ for 0 to 5 min; the reaction was started with 10 μmol/L ATP and allowed to continue for 5 min before it was stopped with 1 mL of 20 % trichloroacetic acid. After centrifugation, aliquots of the supernatant were used for the determination of inorganic phosphorus. The measurement of ATPase activity was performed using a Shimadzu UV-260 recording spectrophotometer at 20℃ and expressed as nmol pi/min/mg of mitochondrial protein.

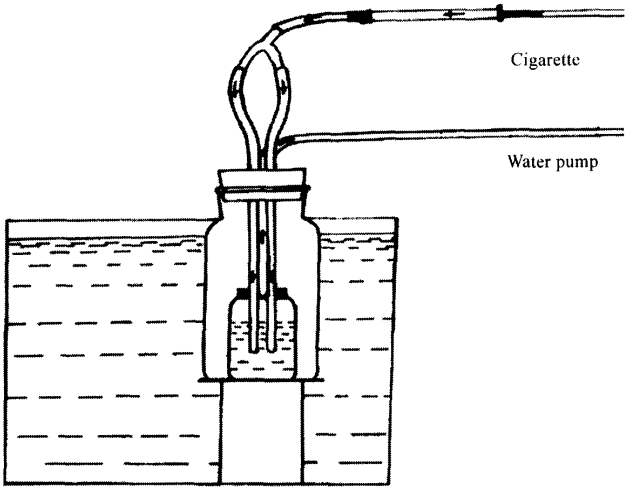

FIG.1. Apparatus used for the preparation of CSE solutions. Cigarette smoke was bubbled through the buffer (10 mmol/L HEPES, pH 7.4) at one cigarette per 5 minutes using a simple three-way pipe with a cigarette inserted into a holder attached to one pipe and the other two pipes going to a 60 mL brown glass bottle with 30 mL buffer in a water bath at 37℃, and the left pipe was driven by a water pump (constant pressure at –5 cm H_2O).

Determination of Cytochrome C Oxidase Activity

Because of rapid autoxidation of cytochrome C at room temperature, the special procedure was needed to prepare fully reduced cytochrome, which contained no excessive reducing agent. Cytochrome C was treated with a tenfold excess of sodium dithionite and immediately desalted through a Sephadex-G25 column in a one mL syringe and pre-equilibrated with the same buffer. The Sephadex G-25 column was then centrifuged at 2 000 g and 4℃ for 3 min to recover the reduced cytochrome C rapidly, which was prepared before use and stored at 4℃.

The mouse brain mitochondrion were exposed to nicotine or CSE at various concentrations in the absence or presence of vitamin C for 60 minutes at 37℃. The reaction was initiated by the addition of the treated mitochondrial protein (0.1 mg/mL). The medium was stirred with a hand-driven plastic rod for 2 seconds to prevent the formation of foam and air bubbles[11]. Cytochrome C oxidase activity was assayed according to the method of Wharton[12]. The decrease in absorbance was measured using a Shimadzu UV-260 spectrophotometer at 550 nm and 540 nm every 15 seconds for 1 min at 20℃. The molar extinction coefficient of cytochrome C was $\Delta\varepsilon = 19$ mmol/L^{-1} cm^{-1} at 550-540 nm.

Assay of Malondialdehyde (MDA) in Mitochondria

The lipid peroxidation of mouse brain mitochondrial membrane was determined as previously described[13]. The formation of thiobarbituric acid reactive substances (TBARS) was quantified in terms of MDA equivalents. The mitochondrial protein concentration and incubation condition were the same as ATPase activity determination. The ferrous-cysteine reaction system including (in mmol/L) 0.2 cysteine and 0.05 $FeSO_4$ was used as the positive free radical generation system to induce mitochondrial lipid peroxidation[14].

Measurement of Member Fluidity in Mitochondria

Ten μL of 1 mmol/L solution of DPH in 0.1% DMSO was added to 1 mL mitochondrial mixture (0.1 mg/mL protein) at the beginning of exposure to nicotine or CSE (0, 4, 8, 16, or 32 μL primary CSE solution/mL) or the ferrous-cysteine reaction system described as above at 37℃ in the dark. After equilibration of DPH fluorescence labelling for 60 min, the fluorescence intensity of the labelled mitochondrial membrane was measured at excitation light wavelength at 365 nm and emission light at 430 nm, and with the slit widths at 5 nm performed on a Hitachi spectrofluorometer at 20℃[15]. The fluorescence intensity of DPH-labelled mitochondria was measured perpendicular (I_t) and parallel (I.p) to the polarization phase of the exciting light. Steady-state fluorescence polarization (P) was calculated with Perrin's equation: $P = (I_p - GI_t)/(I_p + GI_t)$, where G is the correlation factor I_t/I_p.

Lipid Peroxidation of Mitochondrial Membrane and Mitochondrial DNA Peroxidation Detected by Ultra-weak Chemiluminescence Analyzer.

The mitochondrial DNA (mtDNA) was prepared as described by Hayakawa[16]. Briefly, mouse brain mitochondrial fraction was prepared as above. The mitochondrial fraction was resuspended in lyses buffer (10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 0.5% SDS, 2.0 units per mL of proteinase K) and incubated at 37℃ for 2 hours. Then, further purification was made by digestion with 10 μg/mL of pancreas ribonuclease for 30 min. at 37℃. The mtDNA was extracted twice with chloroform/isoamyl alcohol (24:1, v/v). The extracts were mixed (1:15, v/v) with 3 mol/L sodium acetate (pH 7.0) and 2 volumes of 100% cold ethanol to precipitate DNA at -20℃ for 1 h. The samples were centrifuged at 12 000 g for 10 min. The resultant DNA was washed twice with 70% ethanol, air-dried for 3 min and dissolved in 100 μL of TE buffer containing 10 mmol/L Tris, one mmol/L EDTA, pH 7.4. DNA concentration was measured by reading absorbance at 260 nm and its purity was assessed by assuring $A_{260}/A_{280}>1.75$. The samples were used for ultra-weak chemiluminescence measurement with BPLC-ultra-weak chemiluminescence analyzer (Manufactured by Institute of Biophysics, Academia Sinica, Beijing, China.) at 25℃ and 800 V.

The amount of mitochondrial membrane lipid peroxidation (0.5 mg/mL protein) or 8-OH-dG formation in damaged mtDNA (1 μg/mL) was proportional to that of chemiluminescence intensity as measured by the ultra-weak chemiluminescence analyzer. The ferrous-cysteine and $CuSO_4$-Phen-H_2O_2 systems were used as the positive free radical generation systems to induce mitochondrial lipid peroxidation and DNA damage, respectively (Sigman, 1990). The $CuSO_4$-Phen-H_2O_2 reaction mixtures contained (in mmol/L) 0.5 $CuSO_4$, 3.5 mol/L 1, 10-phenanthroline (phen), 3.5 vitamine C, together with mtDNA. And the reaction was started by adding of CSE, or H_2O_2 (6%).

Assay of Mitochondria Swelling

After incubation of mitochondria with isosometric respiratory medium (in mmol/L) 140

KCl, 10 HEPES, pH 7.4 in the absence or presence of nicotine or CSE (4, 8, 16, or 32 μL primary CSE solution/mL) for 60 min at 37℃, the mouse brain mitochondria (0.15 mg/mL) was energized with (in mmol/L) 10 Pi (K) and 10 succinate. The swelling of mitochondria was initiated by adding 250 μmol/L or 6.5 μmol/L $CaCl_2$. The kinetic curves of mitochondrial swelling were monitored via the decrease in absorbance of the mitochondrial suspension at 520 nm for 30 min at 20℃ by a Shimadzu UV-260 spectrophotometer[17].

Mitochondrial Morphological Examination by Electron Microscopy

Incubation conditions for mitochondrial morphological changes were the same as ATPase experiment. The entire reaction systems were centrifuged at 15 000 g for 20 min. The supernatant was removed, and the micropellet was immersed in 4 mL of fixative solution with the composition: 2.5% glutaraldehyde, 140 mmol/L KCl, and 10 mmol/L HEPES, pH 7.4. The pellets were fixed at 4℃ overnight. After rinsing, post-fixation was carried out in 2% osmium tetroxide. The preparations were then dehydrated in alcohol, embedded in Epon and sectioned with a Porter-Blum MT-2 ultramicrotome. Staining was carried out with uranyl acetate in 50% alcohol and lead citrate. The sections were examined with a Hitachi transmission electron microscope[18].

Data Analysis

All data were expressed as $\bar{x}\pm s$. Data differences between groups were analyzed using Student's t test according to different experiments. A value of $P<0.05$ was taken as significant.

RESULTS

Effect of CSE on Mouse Brain Mitochondrial Respiratory Enzyme Activity, MDA Production and Membrane Fluidity

When the mitochondrion were treated with various concentrations of CSE for 60 minutes, the respiratory enzyme activities of ATPase and cytochrome C oxidase were reduced with the concentration of CSE increased (Table 1). At the concentration of 32 μL/mL CSE, both ATPase and cytochrome C oxidase activity were reduced by about 80%. It should be noted that, since our measurements were performed under the same condition, the kinetic changes of the two kinds of enzyme activities might be seen. The magnitude of the decrease in mitochondrial ATPase activity was comparable to that in cytochrome C oxidase activity. However, the decline of cytochrome C oxidase activity was about two times faster than that of ATPase activity induced by the same concentration of CSE. In contrast, MDA production and membrane fluidity expressed in the degree of fluorescence polarization and the indices of mitochondrial membrane lipid peroxidation showed no significant changes when the respiratory enzymes were inhibited. And incubation of vitamin C at various concentrations with mitochondria was ineffective on the prevention of CSE-induced losses of ATPase activity and cytochrome C oxidase activity.

Role of CSE in Ultra-weak Luminescence Generation of Mouse Brain Mitochondria and Mitochondrial DNA

The mouse brain mitochondria or mitochondrial DNA was incubated both in the absence and presence of 32 μL/mL CSE for 1 h and monitored by ultra-weak chemiluminescence

analyzer to detect the generation of chemiluminescence intensity. The addition of CSE had no effect on initiating the production of ultra-weak chemiluminescence of mouse brain mitochondria and mitochondrial DNA. However, the addition of free radical systems (ferrous-cysteine or phen-vitamine C-H_2O_2 free radicals generation system) initiated a significantly steeper and short increase of chemiluminescence, indicating that damage of brain mitochondria by generated oxygen free radicals occurred (Figs. 2 and 3).

TABLE 1

Effect of CSE or Nicotine on Mouse Brain Mitochondrial Cytochrome C Oxidase, ATPase Activities, MDA Production and Membrane Fluidity[a] ($\bar{x} \pm s$)

Group	COX Activity (nmol/min/mg)	ATPase Activity (μmol Pi/min/mg)	MDA (nmol/mg protein)	Degree of Fluorescence Polarization (P)
Normal (0)	450.00±17.46	0.60±0.03	2.82±0.12	0.279±0.017
Nicotine (1 μmol/L)	438.73±8.73	0.61±0.019	2.98±0.32	0.279±0.011
Ferrous-cysteine[b]	-	-	14.63±0.41**	0.259±0.015**
		CSE[c]		
4 μL/mL	125.00±5.04**	0.37±0.01**	2.80±0.60	0.273±0.016
8 μL/mL	103.93±9.95**	0.29±0.02**	3.22±0.16	0.287±0.007
16 μL/mL	78.95±4.30**	0.19±0.01**	3.37±0.18	0.271±0.011
32 μL/mL	84.09±9.44**	0.07±0.01**	3.08±0.13	0.268±0.014
		CSE 8 μL/mL +vitamin C[d]		
10^{-4} mol/L	80.70±4.30	0.23±0.01	2.95±0.19	0.280±0.009
10^{-5} mol/L	113.45±4.30	0.28±0.02	3.10±0.17	0.279±0.012
10^{-6} mol/L	107.89±3.04	0.27±0.03	3.17±0.20	0.274±0.013

Note. [a]: Isolated mitochondria (0.1 mg protein/mL) was added to a total volume of 1 mL in the absence (controls) or presence of increased CSE concentrations. [b]: The ferrous-cysteine reaction system including 0.2 mmol/L cysteine and 0.05 mmol/L $FeSO_4$ was used as the positive control to induce mitochondrial lipid peroxidation. [c]: The primary CSE solution (1 cigarette/30 mL buffer) was diluted into 0, 4, 8, 16 or 32 μL/mL reaction buffer, respectively. [d]: Mitochondria was preincubated with vitamin C at 37°C for 15 min before exposure to CSE 8 μL/mL. **$P<0.01$ Vs normal.

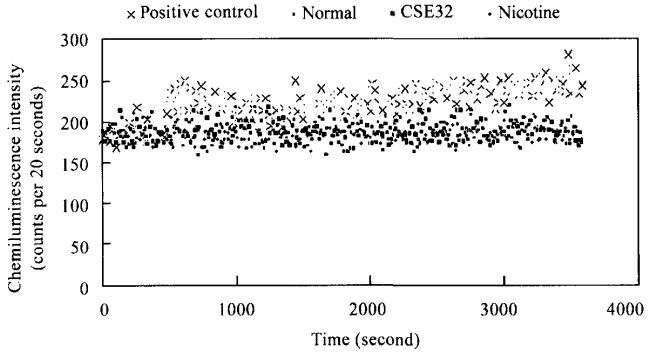

FIG. 2. Detection of the chemiluminescence intensity of mouse brain mitochondria in the presence or absence of CSE or nicotine. Ferrous-cysteine reaction was used to generate free radicals as positive control. Dots represented the individual counts per 20 seconds. Mitochondria (0.5 mg protein/mL) was incubated with CSE or nicotine in the cuvettes at 37℃.

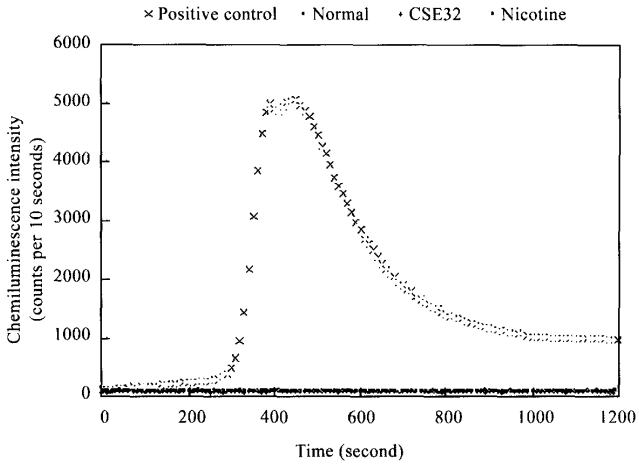

FIG.3. Changes in chemiluminescence properties of mtDNA treated with CSE or nicotine. The mtDNA was incubated in the absence or presence of CSE (32 μL primary CSE solution /mL buffer) for 30 min and the phen-vitamine C-H_2O_2 free radical generation system was used as positive control. Dots represented the individual counts per 10 seconds. The mtDNA concentration was 1 μg/mL at 25℃.

Effect of CSE on Ca^{2+}-induced Swelling of Mouse Brain Mitochondria

The effect of CSE on Ca^{2+}-induced swelling of mouse brain mitochondria varied with the concentration of calcium added. As shown in Fig. 4, CSE showed a dose-dependent inhibitory effect on the mitochondrial swelling response to stimulation of 6.5 μmol/L Ca^{2+}, whereas CSE had a dose-dependent enhancing effect on 250 μmol/L Ca^{2+}-induced mitochondrial swelling (Fig. 5). Moreover, the activity of 6.5 μmol/L Ca^{2+}-induced mitochondrial swelling was higher than that of 250 μmol/L Ca^{2+}.

Addition of 2 mmol/L ADP to the incubation mixture almost completely abolished the stimulating effect of CSE (8 μL/mL) on mitochondrial swelling at the concentration of 250 μmol/L Ca^{2+}. However, the same concentration of ADP had no influence on the inhibitiory effect of CSE on mitochondrial swelling at 6.5 μmol/L Ca^{2+}. Pretreatment of vitamin C was shown to have no effect on 6.5 μmol/L Ca^{2+} and 250 μmol/L Ca^{2+}-induced changes of brain mitochondrial swelling.

Mitochondrial Morphological Examination by Electron Microscopy

Electron microscopic analysis of the mitochondria showed that 8 μL/mL CSE treatment induced inner membrane damage of mouse brain mitochondria and vacuolization of the matrix, whereas the outer mitochondrial membrane appeared to be preserved. Vitamin C showed no protection against alterations of the inner microtubular network. There was no significant morphologic difference between the normal control and the nicotine-treated group (Fig. 6).

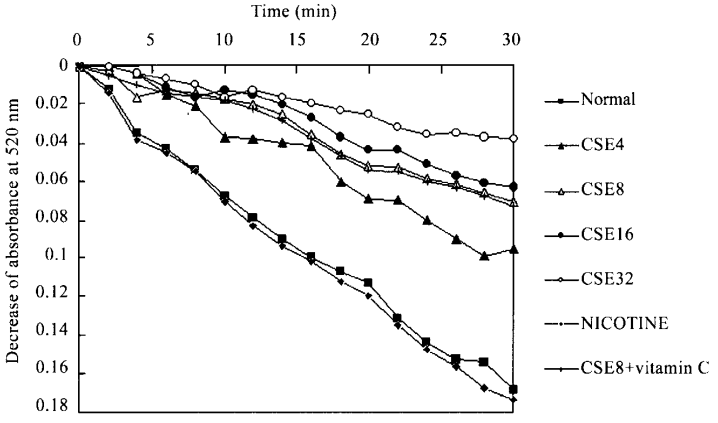

FIG. 4. Effect of CSE or nicotine on 6.5 μmol/L Ca^{2+}-induced mouse brain mitochondrial swelling. Mouse brain mitochondria (0.15 mg protein/mL) was taken at 60 min after incubation with nicotine or various concentrations of CSE. One group was preincubated with vitamin C at 37℃ for 15 min before exposure to CSE 8 μL/mL. Samples were suspended in swelling medium and 10 mM succinate as substrate. Then 6.5 μmol/L $CaCl_2$ was added to initiate mitochondrial swelling, which was monitored via the decrease of absorbance of the mitochondria suspension at 520 nm every two minutes. The figure was a typical recording from three separate experiments.

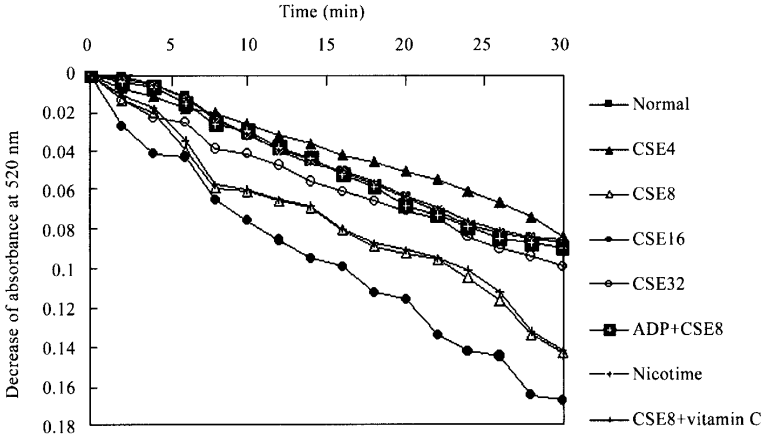

FIG. 5. Effect of CSE or nicotine on 250 μmol/L Ca^{2+}-induced mitochondrial swelling. Samples were taken at 60 min after incubation with nicotine or various concentrations of CSE. The reaction conditions were the same as Fig. 4 except that the swelling was initiated by adding 250 μmol/L $CaCl_2$ in the absence or presence of 2 mmol/L ADP. The traces were typical direct recordings from three separate experiments.

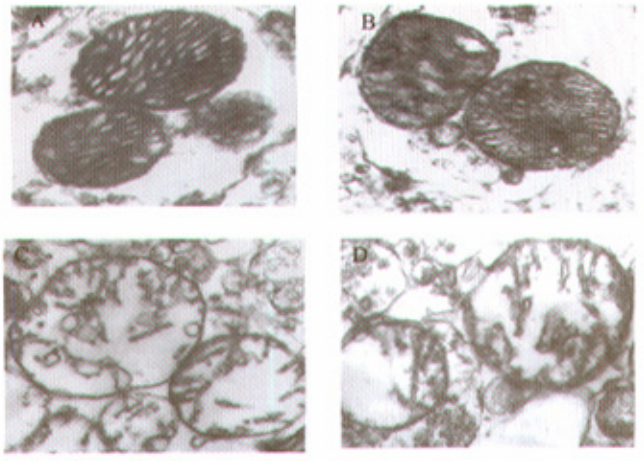

FIG. 6. Electron microscopic characterization of CSE or nicotine-induced changes of mitochondrial morphology. A, normal control; B, nicotine-treated group. Mitochondria (0.15 mg/mL) was treated with CSE (8 μL primary CSE solution/mL) in the absence (C) or presence of 10^{-4} mmol/L vitamin C(D) for 60 min. Magnification (×50 000).

DISCUSSION

The present study demonstrated that CSE decreased the activity of ATPase and cytochrome C oxidase of freshly isolated mouse brain mitochondria in a dose-dependent manner *in vitro*. Moreover, CSE inhibited 6.5 μmol/L Ca^{2+}-induced mitochondrial swelling, which could be indirectly linked to the depression of mitochondrial ATPase and cytochrome C oxidase activity. However, nicotine, the major component of cigarette smoke, showed no significant effect on the activity of ATPase or cytochrome C oxidase. Nicotine may not constitute the toxic component of the CSE that inhibits ATPase and cytochrome C oxidase activity of freshly isolated mouse brain mitochondria.

The free radical-induced oxidative stress has been implicated as an important mechanism responsible for the toxicity of cigarette smoking. Cigarette smoke contains relatively long-lived free radical species detected by direct electron spin resonance technique at supra-physiological concentration *in vitro*[6]. The mitochondrial membrane lipid and DNA, which are the areas predisposed to oxygen free radical damage, are closely related with mitochondrial structure and function[19]. The ultra-weak chemiluminescence spontaneously emitted by mitochondria is closely related to the initiation of oxygen free radicals[20]. In our study, there was no significant change in chemiluminescence of mitochondrial membrane or DNA, membrane lipid fluidity or thiobarbituric acid reactive substances (TBARS) level when mitochondrial respiratory enzyme activity was inhibited. Vitamin C is known to be an effective antioxidant and play an important role in biological oxidation. It intercepts aqueous-phase oxygen free radicals before the initiation of hyperoxidation reaction. Vitamin C is capable of protecting tissues from oxidative damage so as to keep certain enzymes in their reduced states[21]. However, in the present study, vitamin C was not shown to prevent

the inhibition of mitochondrial ATPase, cytochrome C oxidase activity or mitochondrial swelling response induced by Ca^{2+}. Su *et al.*[7] reported that incubation of endothelial cells with CSE resulted in a time- and dose-dependent decrease in nitric oxide synthase activity and content, which was also not prevented by vitamin C or other antioxidants. Therefore, oxidative stress might not be the main cause of brain mitochondria dysfunction induced by CSE. The toxic effect of CSE on brain mitochondria appears to be due to its direct action on enzymatic activity rather than through oxygen free radical injury.

Nakayama *et al.*[22] reported that CSE induced considerable numbers of DNA single-strand breaks in cultured human lung carcinoma cells, which were treated with smoke-PBS (one cigarette/6 mL) for 1 h at 20℃. The CSE concentration used by them was about five hundred times higher than that in our study. It should be pointed out that the primary CSE solutions were adjusted to be comparable to those of human smokers in our study. The CSE at this level was not shown to function as a continuous source of oxygen free radicals detected by the ultra-weak chemiluminescence method. Before cigarette smoke diffused into blood to play a role in tissue and cell injury, it should pass through alveolar respiratory membrane containing 6 layers including liquid rich in surfactant. The oxygen radicals in cigarette smoke could be quenched immediately in the fluid. During the preparation of CSE, the oxygen free radicals might have degraded before they could react with mitochondria in our study. Nevertheless, oxygen free radicals might contribute to the high risk of alveolar and bronchial epithelial injury, which were directly exposed to cigarette smoke.

We also found that CSE enhanced high concentration of 250 μmol/L Ca^{2+} -induced mitochondrial swelling response. How to explain this phenomenon? It was reported that the massive swelling of mitochondria at high concentration of Ca^{2+} was associated with the opening of mitochondrial membrane permeability transition (MPT) pore[23,24]. The MPT was involved in the maintenance of mitochondrial calcium homeostasis to avoid the uptake of calcium by mitochondria and initiation of apoptosis. When the Ca^{2+} concentration in mitochondria sharply increased, especially when adenine nucleotides were depleted, the probability of the open MPT pore would increase sufficiently to show massive swelling response[25]. Our study provided evidence that CSE induced significant inhibition of mitochondrial respiratory enzymes (ATPase and cytochrome c oxidase), and ADP as a MPT inhibitor completely blocked CSE to exacerbate mitochondrial swelling induced by 250 μmol/L Ca^{2+}. Moreover, vitamin C was not shown to protect mitochondria from this swelling response disorder. The electron microscopic observation found the mitochondrial inner membrane was destroyed after exposure to CSE. It is reasonable to speculate that the MPT pore of the mitochondria would be opened by CSE exposure. The opening of mitochondrial MPT pore might be a potential mechanism for mitochondrial damage induced by CSE.

In summary, CSE could induce damage of the structure and function of mouse brain mitochondria *in vitro*, yet no lipid peroxidation phenomena or oxygen free radical generation were observed. Vitamin C could not protect mitochondria from CSE-induced inhibition of mitochondrial respiratory enzyme and Ca^{2+} induced swelling response of mitochondria. Nicotine was not the principle substance responsible for the CSE toxicity. These findings may help to explain the high risk of brain mitochondrial disorder in cigarette smokers. Further study is needed to explore the related mechanism of CSE-induced brain mitochondrial damage.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Chenghe Li and Ms. Yan Liu in the Department of Pathology for their support in the examination of mitochondrial morphology by electron microscopy.

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(*Received January* 26, 2003 *Accepted April* 2, 2003)