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Acute nitrogen dioxide inhalation induces mitochondrial dysfunction in rat brain

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ABSTRACT

Recent epidemiological literatures imply that NO_2 is a potential risk factor of neurological disorders. Whereas, the pathogenesis of various neurological diseases has been confirmed correlate to mitochondrial dysfunction, and mitochondria play the crucial roles in energy metabolism, free radicals production and apoptosis triggering in response to neuronal injury. Therefore, to clarify the possible mechanisms for NO_2-induced neurotoxicity, in the present study, we investigated the possible effects of acute NO_2 inhalation (5, 10 and 20 mg/m^3 with 5 h/day for 7 days) on energy metabolism and biogenesis in rat cortex, mainly including mitochondrial ultrastructure, mitochondrial membrane potential, cytochrome c oxidase activity, cytochrome c oxidase (CO) and ATP synthase subunits, ATP content, and transcription factors. The results showed that NO_2 exposure induced mitochondrial morphological changes in rat cortex, and the alteration was coupled with the abnormality of mitochondrial energy metabolism, including decreased respiratory complexes, reduced ATP production and increased production of ROS. Also, increased ROS in turn caused mitochondrial membrane damage, energy production defect and mitochondrial biogenesis inhibition. It suggests the significantly damaged mitochondrial energy metabolism and impaired biogenesis in rat brain after NO_2 exposure, and provides a new understanding of the pathophysiological mechanisms of NO_2-induced neurological disorders.

1. Introduction

As a byproduct of economic prosperity, the air pollution issue in China has raised more and more attention in recent years, especially for which potential impact on human health, weather and climate. Known as the massive anthropogenic emission, NO_2 was one of the most important pollutants in the aspect of environmental pollution (Akimoto, 2003). In China, the one-hour average concentration limit of national air quality secondary standards for NO_2 is 0.24 mg/ m^3 (GB3095-1996). However, the level of NO_2 can markedly exceed that standard outdoor one particularly in during heavy traffic or rush hours (Chan and Yao, 2008). Higher concentrations are found indoors with a peak level exceeding 2 parts per million (ppm) (Pathmanathan et al., 2003). Also, NO_2 level can even reach up to 4 ppm in garages, ferries, skating ice rinks, or kitchens with gas cookers (Persinger et al., 2002; Frampto et al., 2002; Hussain et al., 2004). Considering its widely diffusion and strong oxidization, NO_2 has arrested a greatly increasing concern on public health. Among these, in addition to traditional respiratory injuries, epidemiological evidence and recent toxicological studies refer to the link between NO_2 exposure and the increasing risk of cardiovascular diseases and neurological disorders, such as ischemic stroke, vascular dementia, autism spectrum disorder, neurodevelopmental impairment and cognitive deficit (Zhu et al., 2012; Li and Xin, 2013; Jung et al., 2013; Kim et al., 2014; Morales et al., 2009), which implies that NO_2 inhalation targets brain and leads to neuronal impairment.

Oxidative stress, occurring in the affected brain regions of several neurological diseases, is considered to be one of the most probable molecular mechanisms for neuronal brain injury; and the generation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) in these disorders may play an important role (Calabrese et al., 2005). Mitochondria are considered to be the major intracellular source of ROS, and mitochondria-generated ROS are involved in several physiological signaling cascades (Valko et al., 2007; Dröge, 2002; Giorgio et al., 2007; Afanas'ev, 2007). Mitochondrial dysfunction promotes an overproduction of ROS, while excessive ROS-induced cellular injury will in turn target mitochondria. Mitochondrion is an important organelle required for bioenergetic processes and also plays a key role in energy production, cell metabolism, calcium homeostasis, free radicals production, or apoptosis triggering (Achanta et al., 2005; Picone

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et al., 2014; Hoppins, 2014). In the brain, where the request and consumption of energy is in the highest level, mitochondrial impairment could be a serious threat for neurons survival; and the pathogenesis of various neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and Friedreich ataxia may be involved in the mitochondrial dysfunction (Picone et al., 2014).

NO_2 exposure on uinea-pigs showed a significant elevation of the rate of lipid peroxidation in brain areas (Farahani and Hasan, 1990). Recent study confirmed that NO_2 could exert oxidative stress to rat brain with altered antioxidants activity including superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Li et al., 2012). A variety of studies have implicated that mitochondrial functions can be negatively affected by environmental toxicants stimulus, such as SO_2, $PM_{2.5}$, black carbon and O_3 (Qin et al., 2012; Li et al., 2003; Meyer et al., 2013; Colicino et al., 2014; Lee, 1968). Therefore, we hypothesized that mitochondrial injury and dysfunction may be important mechanism contributing to neurological disorders following NO_2 inhalation. To test the implication, in the present study, we acutely exposed male Wistar rats to NO_2 5, 10 and 20 mg/m^3, and investigated the possible effects on energy metabolism and biogenesis in brain mitochondrial responses, mainly including mitochondrial ultrustructure, mitochondrial membrane potential, the respiratory chain complexes (RCC), ATP content, and transcription factors. The work was aimed to clarify the possible mechanistic basis for exploring an association between NO_2 inhalation and increased risk for neurological disorders and opening up therapeutic approaches of treating, ameliorating, or preventing brain injuries resulting from NO_2 exposure in atmospheric polluting environment.

2. Materials and methods

2.1. Animals and treatment protocols

Male Wistar rats, weighing 160–180 g, were divided randomly into four equal groups, each group containing 6 male rats. The rats were purchased from Experimental Animal Center, Academy of Military Medical Sciences of Chinese PLA (Beijing, China).The rats were routinely screened for common rat pathogens. The rats used in these experiments were housed in rooms determined to be free of common pathogens. The animals were housed in groups of 6 rats in stainless steel cages under standard conditions (24 ± 2 °C and $50 \pm 5\%$ humidity) with a 12 h light–dark cycle. Three groups were exposed to 5.04 ± 0.15, 9.96 ± 0.22 and 19.78 ± 0.13 mg/m^3 NO_2 in 1 m^3 exposure chambers for 5 h/day for 7 days, respectively. The choice of concentration stated in our previous studies (Li et al., 2012). The fourth, control group was exposed to filtered air in another 1 m^3 chamber using the same schedule. NO_2 gas for exposure was purchased from the Yihong Gas Industry Co., Ltd., Taiyuan, China and the purity is up to 99.99%.The atmosphere of NO_2 in the exposure chamber was obtained by mixing during 5 h continuously at a constant flow rate with the filtered ambient air pumped to the chamber at a flow rate of 30 L/min. The NO_2 was diluted with fresh air at the intake port of the chamber to yield the desired NO_2 concentrations. The NO_2 within the chambers was measured every 30 min by the Saltzman colorimetric method using a spectrometer calibrated at 540 nm (Kumie et al., 2009). When not being treated, the rats had free access to food and water. Rats were decapitated 18 h after the last exposure. Brain were isolated quickly, frozen in liquid nitrogen and then stored at −80 °C until use.

2.2. Mitochondrial MTT and JC-1 assay

The fresh separated brain cortices were kept on ice and washed with cold extraction buffer (10 mM HEPES, pH 7.5, containing 1 M mannitol, 350 mM sucrose, and 5 mM EGTA) and minced in small fragments. The extraction buffer containing bovine serum albumin (BSA) was added and homogenized. The homogenates were centrifuged at *600g*, 5 min. And the supernatant liquids were transferred into a new tube and centrifuged at 11,*000g*, 10 min. The pellets were resuspended in extraction buffer and centrifuged again. The last pellets were suspended in store buffer (10 mM HEPES, pH 7.5, containing 1.25 M sucrose, 5 mM ATP, 0.4 mM ADP, 25 mM sodium succinate, 10 mM K2HPO4, and 5 mM DTT). Protein concentration was determined by the Bradford method (Bradford, 1976). Freshly isolated mitochondria were either used immediately for JC-1 and MTT assays or stored at −80 °C.

The MTT assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-H-tetrazolium bromide (MTT) by hydrogenase activity in functionally intact mitochondria. The MTT reduction rate was used to assess the activity of the mitochondrial respiratory chain in isolated mitochondria by the method of Liu et al. (1997). Briefly, 100 μg mitochondrial sample was incubated with 20 μl MTT (5 mg/ml) for 30 min at 37 °C. The blue formazan crystals were solubilized with dimethylsulfoxide and quantified spectrophotometrically in a microplate reader using a 565 nm filter (Bio-Rad, Milano, Italy). Mitochondrial membrane potential (MMP) was assessed in brain mitochondria using the lipophilic cationic probe JC-1. Briefly, a mitochondrial suspension was diluted with assay buffer (20 mM MOPS, pH 7.5, containing 110 mM KCl, 10 mM ATP, 10 mM $MgCl_2$, 10 mM sodium succinate, and 1 mM EGTA) to 0.5 mg/mL. The samples were incubated in 0.2 μg/mL JC-1 solution at 37 °C for 20 min followed by fluorescence measurement at 490 nm excitation, and 590 nm emission. MMP was expressed as the fluorescence units (FLU)/mg pro.

2.3. Measurement of cytochrome c oxidase activity

Cytochrome c oxidase activity was determined using a Cytochrome c Oxidase Assay kit (Sigma-Aldrich, St. Louis, MO). Determination of cytochrome c oxidase activity was based on a colorimetric assay that quantifies the oxidation of ferrocytochrome c to ferricytochrome c via cytochrome c oxidase, a reaction that results in a decrease in absorbance at 550 nm. The decrease in absorbance at 550 nm was monitored by a spectrophotometer (U3010, Hitachi, Japan) calibrated to zero using the assay buffer (10 mM Tris–HCl and 120 mM KCl, pH 7.0). In a cuvette, 950 μL of assay buffer was combined with 90 μL of enzyme buffer (10 mM Tris–HCl and 250 mM sucrose, pH 7.0) and 10 μL of isolated mitochondria. The reaction was initiated by the addition of 50 μL of ferrocytochrome c (reduced with 0.1 M dithiothreitol), and the decrease in absorbance at 550 nm was measured for 1 min. Activity was calculated based on our previous study (Qin et al., 2012).

2.4. Assay of ATP

The amount of ATP was measured by the luciferinluciferase method following the protocol of ATP detection kit (Beyotime, China). About 50 mg cortical tissues were homogenized with 500 μL lysis buffer from the ATP detection kit. After centrifuged at 12,*000g* for 10 min at 4 °C, the supernatant was transferred to a new tube for ATP test. The luminescence from a 100 μL sample was assayed in a Varioskan Flash Multimode Reader (Thermo Scientific, Vantaa, Finland) together with 100 μL ATP detection buffer from the ATP detection kit. The standard curve of ATP concentration was prepared from a know amount (1 nM–1 μM).