Toxicology 337 (2015) 21–29

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Cognitive deficits induced by multi-walled carbon nanotubes via the autophagic pathway

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ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form 26 August 2015
Accepted 26 August 2015
Available online xxx

Keywords:
MWCNTs
Cognitive deficits
Autophagy
Chloroquine
Rats

ABSTRACT

Multi-walled carbon nanotubes (MWCNTs) have shown potential applications in many fields, especially in the field of biomedicine. Several studies have reported that MWCNTs induce apoptosis and oxidative damage in nerve cells during *in vitro* experiments. However, there are few studies focused on the neurotoxicity of MWCNTs used *in vivo*. Many studies have reported that autophagy, a cellular stress response to degrade damaged cell components, can be activated by diverse nanoparticles. In this study, we investigated the neurotoxic effects of MWCNTs on hippocampal synaptic plasticity and spatial cognition in rats. Then, we used an inhibitor of autophagy called chloroquine (CQ) to examine whether autophagy plays an important role in hippocampal synaptic plasticity, since this was damaged by MWCNTs. In this study, adult male Wister rats were randomly divided into three groups: a control group, a group treated with MWCNTs (2.5 mg/kg/day) and a group treated with MWCNTs + CQ (20 mg/kg/day). After two-weeks of intraperitoneal (i.p.) injections, rats were subjected to the Morris water maze (MWM) test, and the long-term potentiation (LTP) and other biochemical parameters were determined. Results showed that MWCNTs could induce cognitive deficits, histopathological alteration and changes of autophagy level (increased the ratio of LC3 II /LC3 I and the expression of Beclin-1). Furthermore, we found that CQ could suppress MWCNTs-induced autophagic flux and partly rescue the synapse deficits, which occurred with the down-regulation of NR2B (a subunit of NMDA receptor) and synaptophysin (SYP) in the hippocampus. Our results suggest that MWCNTs could induce cognitive deficits *in vivo* via the increased autophagic levels, and provide a potential strategy to avoid the adverse effects of MWCNTs.

1. Introduction

Nanomaterials have shown increasing usage worldwide since their emergence a few decades ago. Carbon nanotubes (CNTs) are one of the best known nanomaterials due to their unique chemical and physical characteristics (Nakashima and Fujigaya, 2007; Wang et al., 2007). CNTs have shown increasing promise in the field of biomedicine in recent years. CNTs, especially multi-walled carbon nanotubes (MWCNTs), have many applications in medical neuroscience, including as drug carriers (Bianco et al., 2005; Yang et al., 2009), in electrical nerve stimulation (Keefer et al., 2008), and as substrates for nerve cell growth and differentiation (Chao et al., 2009; Chao et al., 2010; Sorkin et al., 2006). Moreover, CNTs have been used to deliver drugs and genetic material into nerve cells in the brain for the treatment of glioma and neurodegenerative diseases because of their ability to pass through cell membranes (Ren et al., 2012; Yang et al., 2010b). Because they have such widespread applications, it is necessary to explore the potential risks that CNTs might pose to human health. A number of studies performed with CNTs have evaluated their toxicity to different organs, such as the lungs, kidneys, and liver (Awasthi et al., 2013; Deng et al., 2009; Li et al., 2007). Several *in vitro* studies have confirmed that CNTs could generate neurotoxic effects, including decreasing cell activity (Belyanskaya et al., 2009; Zhang et al., 2010). In addition, our previous studies have showed that MWCNTs induce cytotoxicity in C6 cells (Han et al., 2012) and inhibit CA1 glutamatergic synaptic transmission in rat hippocampal slices *in vitro* (Chen et al., 2014). However, there is no accurate conclusion about the impaction of CNTs on the nervous system *in vivo*.

Autophagy, which is a highly conserved lysosomal degradation pathway, plays an important role in maintaining cytoplasmichomeostasis (Mizushima and Komatsu, 2011). It is a dynamic

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http://dx.doi.org/10.1016/j.tox.2015.08.011
0300-483X/

physiological process that is necessary for cellular health and survival (Kroemer et al., 2010). Damaged and aging cells or organelles are degraded by autophagy. The double membraned autophagosomes, which can deliver cytosolic components to the lysosome for degradation and recycling, are formed following the activation of the autophagic pathway (Mizushima, 2007). The inactive cytoplasmic LC3 (LC3 I), which is a soluble protein distributed ubiquitously in mammalian tissues and cultured cells, can be converted into the active membranous LC3 II, which triggers the formation of the autophagic vesicle (Mizushima et al., 2010). Thus the ratio of LC3 II/LC3 I is widely used to estimate the level of autophagy. Furthermore, another autophagy-related (Atg) gene Beclin 1 is necessary for the localization of autophagic proteins to a pre-autophagosomal structure. Overexpression of Beclin-1 can improve the level of autophagy activity by interacting with the class III type phosphoinositide 3-kinase (PI3KC3)/Vps34 (Kang et al., 2011). It is well known that autophagy protects cells against accidental death (Kaushik et al., 2011). Indeed, many studies have shown that physiological autophagy is responsible for the survival of neurons (Poels et al., 2012). Cell death that displays the typical features of autophagy such as a massive cytoplasmic vacuolization is defined as "autophagic cell death"(Shen et al., 2012). Recently, some nanoparticles have been regarded as autophagy activators, such as gold nanoparticles (Ma et al., 2011) and TiO_2 nanoparticles (Kenzaoui et al., 2012). Also, reports have indicated that disordered autophagy could disrupt the flow of pre-synaptic terminals and cause axonal dystrophy (Sanchez-Varo et al., 2012). However few reports have illuminated the relationship between synaptic plasticity and the autophagy of neurons caused by MWCNTs.

In this study, behavioral changes, electrophysiological tests and biochemical indexes were used to determine changes of synaptic plasticity after exposure to MWCNTs. We investigated whether MWCNTs could contribute to autophagy enhancement and synaptic plasticity impairment in the CA1 area *in vivo*, and we explored the relationship between autophagic flux and the synaptic plasticity damage caused by MWCNTs. We proved that CQ could prevent MWCNTs-induced synaptic impairment by down-regulating autophagy. These results may reveal a key mechanism of autophagy in the nervous system under MWCNTs treatment, and give experimental basis for the safety of biomedical applications of MWCNTs. Our observation may provide a new potential therapeutic method to relieve synaptic impairment induced by MWCNTs.

2. Materials and methods

2.1. Materials and reagents

The MWCNTs used in this study were obtained from the Institute of Metal Research, China Academy. The average length of MWCNTs was approximately 2 μm and the diameter was approximately 10–20 nm. The MWCNTs were suspended in 0.9% NaCl with 0.1% Tween 80, and the suspensions were sonicated for 20 min before each use. The characteristics of the nanoparticles used in this study can be found in our previous study (Han et al., 2012).

Anti-NMDAR2B antibody and anti-Synaptophysin antibody were purchased from Abcam (Cambridge, UK). Anti-LC3 antibody was obtained from MBL (Nagoya, Japan). Anti-Beclin-1 antibody was purchased from Cell Signaling Technology (MA, USA). Anti-β-actin antibody was purchased from Santa Cruz Biotechnology, Inc. CA (California, USA).

2.2. Animals and treatment

Specific-pathogen free (SPF) adult male Wistar rats, weighing 200–220 g, were purchased from the Experimental Animal Center of the Chinese Academy of Medical Science, and reared in the Animal House of Medical School in Nankai University. Conditions were kept at 22 ± 2 °C, and rats were housed in pairs in clear plastic cages on a 12:12 h light/dark cycle with *ad libitum* access to food and water. All experiments were performed according to protocols approved by the Committee for Animal Care at Nankai University and in accordance with the practices outlined in the NIH Guide for the Care and Use of Laboratory Animals. Rats were acclimated for one week before exposure.

Animals were randomly divided into three groups, a control group ($n = 8$), a MWCNTs-treated group ($n = 8$) and a MWCNTs + CQ group ($n = 8$). In the MWCNT group, rats were treated with MWCNTs at a dose of 2.5 mg/kg (Muller et al., 2005) via intraperitoneal (i.p.) injection once per day over 14 consecutive days. Rats in the MWCNTs + CQ group were i.p. injected with CQ (20 mg/kg/d, Wako Pure Chemical Industries, Ltd., Osaka, Japan) (Maeda et al., 2013) dissolved in a suspension, 30 min before the MWCNTs injection, while the animals in the control group received the same dose of only the suspension without CQ.

2.3. Physical observation

Each rat was weighed and recorded every two days at the same time over the 14 days.

2.4. Morris water maze test

After 14 days of treatment, all rats of every group were trained and tested with the Morris water maze (MWM, RB-100A type, Beijing, China) to monitor their spatial learning and memory behaviors. This system involves a circular tub (height 60 cm, diameter 150 cm) and a device, which is connected to a personal computer to capture the rat's swimming pathway. The maze was filled with water maintained at 25 ± 1 °C and dyed by nontoxic black ink. The water was divided into four equal quadrants (I–IV), and a 10-cm-diameter platform, whose surface was 1.5–2 cm below the water surface, was positioned in the middle of quadrant III.

The test consisted of two consecutive stages, initial training and re-acquisition training. Each stage included two phases called the place navigation phase and the spatial probe phase. During the navigation phase, rats were subjected to two sessions (each session consisted of four trials) of training per day for five consecutive days. In each trial, the animals were gently put into the water from a random point of the quadrant. The rats were given 60 s to learn to find the hidden platform. During this stage, the time it took for rats to find the platform (escape latency) and the swimming speed were recorded. If a rat failed to locate the platform within 60 s, it was guided by the experimenter to stay on the platform for 10 s, and its escape latency was recorded as 60 s. The interval between each of the trials was approximately 10 min. The order of starting points was the same for all animals. The rats were given the spatial probe trial test 24 h after the last trial of the navigation phase. The platform was removed during the spatial probe phase. Rats were released individually into water from the starting point of quadrant I and allowed to swim freely for 60 s. Only one trial was carried out in this phase. Quadrant dwell time (the percentage of time spent in the target quadrant) and platform crossings (numbers of times the rat passed the platform area) were measured. After that, re-acquisition training was performed immediately to examine the learning flexibility. The methods used and parameters recorded were the same as those in place navigation phase and spatial probe phase except the platform was moved to the contra lateral quadrant.

2.5. In vivo *electrophysiological testing*

The LTP and depotentiation were measured after rats had undergone the MWM test. The protocols used were similar to