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Research article

Methane rescues retinal ganglion cells and limits retinal mitochondrial dysfunction following optic nerve crush

Ruobing Wang [a, 1], Qinglei Sun [a, 1], Fangzhou Xia [a, 1], Zeli Chen [a], Jiangchun Wu [a], Yuelu Zhang [a], Jiajun Xu [b, **], Lin Liu [a, *]

[a] Department of Ophthalmology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China
[b] Department of Anatomy, Second Military Medical University, Shanghai, China

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ABSTRACT

Secondary degeneration is a common event in traumatic central nervous system disorders, which involves neuronal apoptosis and mitochondrial dysfunction. Exogenous methane exerts the therapeutic effects in many organ injury. Our study aims to investigate the potential neuroprotection of methane in a rat model of optic nerve crush (ONC). Adult male Sprague-Dawley rats were subjected to ONC and administrated intraperitoneally with methane-saturated or normal saline (10 ml/kg) once per day for one week after ONC. The retinal ganglion cells (RGCs) density was assessed by hematoxylin and eosin staining and Fluoro-Gold retrogradely labeling. Visual function was evaluated by flash visual evoked potentials (FVEP). The retinal apoptosis was measured by terminal-deoxy-transferase-mediated dUTP nick end labeling (TUNEL) assay and the expression of apoptosis-related factors, such as phosphorylated Bcl-2-associated death promoter (pBAD), phosphorylated glycogen synthase kinase-3β (pGSK-3β), Bcl-2 associated X protein (Bax) and Bcl-2 extra large (Bcl-xL). Retinal mitochondrial function was assessed by the mRNA expressions of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM), the mitochondrial DNA (mtDNA) copy number, citrate synthase activity and ATP content. Methane treatment significantly improved the RGC loss and visual dysfunction following ONC. As expected, methane also remarkably inhibited the retinal neural apoptosis, such as the fewer TUNEL-positive cells in ganglion cell layer, accompanied by the up-regulations of anti-apoptotic factors (pGSK-3β, pBAD, Bcl-xL) and the down-regulation of pro-apoptotic factor (Bax). Furthermore, methane treatment suppressed up-regulations of critical mitochondrial components (PGC-1α, NRF1 and TFAM) mRNA and mtDNA copy number, as well as improved the reduction of functional mitochondria markers, including citrate synthase activity and ATP content, in retinas with ONC. Taken together, methane treatment promotes RGC survival and limits retinal mitochondrial dysfunction against ONC insult. Methane can be a potential neuroprotective agent for traumatic and glaucomatous neurodegeneration.

1. Introduction

Axonal degeneration occurs commonly in many neurodegenerative and traumatic neurological disorders (Coleman, 2005; Lingor et al., 2012). The optic nerve, as a white-matter tract of the diencephalon composed principally of the axons of retinal ganglion cells (RGCs), is among the most easily accessible parts of the central nervous system (London et al., 2013). Optic nerve crush (ONC) model mimics very closely the damage in traumatic optic neuropathy, causing a partial loss of optic nerve function and secondary loss of ganglion cells (Goldblum and Mittag, 2002). Therefore, the optic nerve injury is the prime model to investigate axonal degeneration and regeneration, due to its anatomical accessibility and clinical relevance (Knöferle et al., 2010). Abundant researches focused on the therapeutic strategies to reduce early neuronal death and inhibit the subsequent apoptosis progress due to the limitation of neural regeneration.

Methane, known as a classic sort of efficient fuel and a major constituent of greenhouse gas ranked second to carbon dioxide, is

* Corresponding author. 160 Pujian Road, Shanghai 200127, China.
** Corresponding author. 800 Xiangyin Road, Shanghai 200433, China.
E-mail addresses: xujiajun1963@126.com (J. Xu), liulin1963rj@126.com (L. Liu).
[1] These authors contributed equally to this work.

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the most simplest organic molecule in the atmosphere. Recent researches paid much attention to its biological and therapeutic role (Liu et al., 2012). Boros et al. found that exhaled methane exerted the protective effects against intestinal ischemia/reperfusion injury (IRI)-induced inflammation (Boros et al., 2012). Thereafter, more researches demonstrated its therapeutic treatments against ischemia/reperfusion (I/R) injury in liver, heart and abdominal skin flaps) (Chen et al., 2015; Song et al., 2015; Ye et al., 2015) and autoimmune hepatitis (He et al., 2016). Surprisingly, methane treatment even exerts a neuroprotective role against carbon monoxide poisoning (Fan et al., 2016) and diabetic retinopathy (Wu et al., 2015). Due to the simplicity, availability, volatility and effectiveness of methane, methane may be a new 'gasotransmitter' candidate (Boros et al., 2015). Although the intracellular targets of methane remains elusive, inhaled methane can improve the hepatic IRI-induced mitochondrial dysfunction (Strifler et al., 2016). Mitochondria are biosynthetic factories that perform various physiological roles, such as energy production, calcium homeostasis and intrinsic apoptosis, that may be targets of methane administration (Dougherty et al., 1967; Carlisle et al., 2005). In addition, due to its tremendous amounts of energy consuming, nervous tissue is particularly susceptible to perturbations in mitochondrial function. Therefore, mitochondrial dysfunction is the underlying cause of many neurodegenerative disorders.

Thus, the present study examined the hypothesis that methane treatment exerts a neuroprotective effect on RGC in a rat model of ONC and explored the potential mechanisms whether methane administration improves retinal mitochondrial dysfunction following acute axonal damage.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats weighing 200–220 g (Shanghai Slac Laboratory Animal CO. LTD, Shanghai, China) were used in the experiment, housed at 22 °C, with a 12:12 h light/dark cycle controlled environment and sufficient food and water. The experiment was well performed according to the Guide for the Care and Use of Laboratory Animals recommended by the National Institutes of Health and approved by the Ethics Committee for Animal Experimentation of Shanghai Jiao Tong University. Adequate anesthesia/analgesia was made to minimize suffering in all procedures.

2.2. Preparations of methane-saturated saline (MSS)

According to previous study (Wu et al., 2015), methane was dissolved in normal saline (NS) and then pressured at 0.4 MPa for 8 h to reach a supersaturated level. MSS was stored at 4 °C for 1 week at most and freshly made one day before ONC surgery to guarantee the high concentration of methane. Before using, MSS was placed in normotheric situation until it was room-temperatured. A gas chromatography (Gas Chromatography-9860, Qiyang, Shanghai, China) was applied to measured the concentration of methane in saline. The concentration of MSS made in such way could last for a long time and could also be detected in rats injected with such saline by blood as previous study described (Ye et al., 2015).

2.3. ONC surgery and methane treatment

The rats were intraperitoneally anesthetized with 10% chloral hydrate (4 ml/kg). A lateral canthotomy was applied in the superior orbital rim of the right eye under a microscope. The optic nerve was exposed as the previous study described (Biermann et al., 2010) and crushed 2 mm away from the globe for 10 s through a Yasargil aneurysm clip carefully without damaging any small vessels (Maeda et al., 2004; Sarikcioglu et al., 2007). Then the eyelids were sutured after the operation. Ampicillin was applied to the wound to prevent additional infection and a direct ophthalmoscope was applied to ensure the integrity of the retinal blood supply. After ONC surgery, rats were injected intraperitoneally with MSS or NS (10 ml/kg) once per day in the following one week. Controls also received intraperitoneal injection with MSS or NS following sham surgery. Therefore, the following four groups were composed: (1) Sham group; (2) ONC group; (3) ONC + Methane group; (4) Sham + Methane group. Fig. 1 summarizes the schematic representation of experimental setup and further analysis. Table 1 summarizes the number of rats used in each group according to the study design.

2.4. Hematoxylin and eosin (H&E) staining and observation

At two weeks after the operation, each removed eye was marked at the top with a string for further location. Fixed in 4% paraformaldehyde and embedded in paraffin, the eyes from six rats per group were stained with H&E as previous study (Wang et al., 2010). Each section was sliced within 4 μm. The sections were selected under a microscope where the optic nerve head and marking string were to ensure the section spacing. The retinas were imaged and analyzed by Imaging-Pro-Plus software (DSLR Remote Pro). The number of cells in ganglion cell layer (GCL) were observed and calculated in 10 high powered fields (HPF) at 400 × magnification on one slide from each eye.

2.5. Retrogradely labeling of RGCs with FluoroGold

To distinguish RGCs from dye-engulfing macrophages and microglia, the retrograde labeling of RGCs was performed at one week before ONC surgery in six rats per group (Levkovitch-Verbin et al., 2003; Wang et al., 2010). Under anesthesia, the 1.5 μL of 5% of FluoroGold (Sigma-Aldrich Co., St. Louis, MO) was injected stereotactically into the colliculus superior at 1 week prior to ONC surgery. The FluoroGold-positive RGCs was counted in retinal flatted mounts under a fluorescence microscope at 400 × magnification at two weeks after ONC. The number of RGCs in the flat-mounted retina was counted with a fluorescence microscope. At least three retinal preparations were analyzed for each sample.

2.6. Flash visual evoked potentials (FVEP)

At two weeks after the operation, FVEP were applied in six rats per group for an evaluation of visual function with a evoked potential analyzing instrument (BMLab 4.0, Second Military Medical University, Shanghai, China). The other eye must be covered while the operated eye was being tested. Rats were fixed into a stereotaxic frame in a dark background. A drill was used to open skulls and expose visual cortices. Then two active electrodes were placed on the pachymeninx and skin of interorbital region. While testing, a single flash with rate of 2.0 Hz and average of 50 sweeps were regulated. The rejecting artifact threshold was set at 50 mv, and a sample rate of 2000 Hz. The latency and amplitude of the first negative N1 and positive P1 peaks of FVEP were recorded to be further analyzed (Wang et al., 2010).

2.7. Terminal-deoxy-transferase-mediated dUTP nick end labeling (TUNEL) assay

The apoptotic cells were determined following TUNEL staining