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Effects of realgar on GSH synthesis in the mouse hippocampus: Involvement of system X_{AG}^-, system X_C^-, MRP-1 and Nrf2

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ABSTRACT

Realgar is a type of mineral drug that contains arsenic and has neurotoxicity. Glutathione (GSH), which is the main antioxidant in the central nervous system, plays a key role in antioxidant defenses and the detoxification of arsenic. However, whether realgar interferes with the synthesis of GSH in the brain and the molecular mechanisms underlying its effects are largely unknown. Here, we used mouse models of exposure to realgar to show that realgar affects the synthesis of GSH in the hippocampus, leading to ultrastructural changes in hippocampal neurons and synapses and deficiencies in cognitive abilities, and that the mechanisms that cause this effect may be associated with alterations in the expression of system X_{AG}^-, system X_C^-, multidrug resistance-associated protein 1 (MRP-1), nuclear factor E2-related factor 2 (Nrf2), γ-glutamylcysteine synthetase (γ-GCS), and the levels of glutamate (Glu) and cysteine (Cys) in the extracellular fluid. These findings provide a theoretical basis for preventing the drug-induced chronic arsenic poisoning in the nervous system that is triggered by realgar.

1. Introduction

Realgar, as a type of mineral drug that contains arsenic, has been used in traditional Chinese medicines (TCMs) for thousands of years in China. A number of realgar-induced chronic arsenic poisoning cases have been reported in the past decades as a result of abuse of the drug by clinics (Liu et al., 2008). Therefore, there is heightened concern regarding the neurotoxic effects of realgar on humans. Epidemiological studies have shown that long-term exposure to arsenic can result in impairments in superior neurological functions, such as cognitive, learning and memory functions (Sun, 2004; Von Ehrenstein et al., 2007). Animal in vivo and vitro experiments have also confirmed that arsenic interferes with synaptic transmission, affects long-term potentiation, and causes learning and memory deficits (Sharma and Sharma, 2013; Krüger et al., 2009). In our previous study, we found that realgar also caused impairments in spatial learning and recognition memory (Huo et al., 2015). A substantial amount of data suggests that arsenic influences redox homeostasis in the body and decreases GSH levels and antioxidant enzyme activity, ultimately resulting in oxidative damage to brain tissues and subsequent abnormal neuronal apoptosis (Chen et al., 2015; Flora, 2011). However, whether and how realgar causes changes in brain GSH levels remains unknown.

Glutathione (GSH) is a free radical scavenger that is involved in maintaining normal functions in cells, and it has a very important protective effect on the nervous system. GSH is produced from three amino acids, including glutamate (Glu), cysteine (Cys) and glycine (Gly), and is catalyzed by γ-glutamylcysteine synthetase (γ-GCS) in neurons. γ-GCS activity is the rate-limiting factor in GSH synthesis, and it is regulated by nuclear factor E2-related factor 2 (Nrf2) (Wild et al., 1999).

Excitatory amino acid transporters (EAATs, also known as system X_{AG}^-) and cystine/glutamate antiporter (also called system X_C^-) transport Glu, Cys and cystine (Cys_2) into cells and play important roles in GSH synthesis (Robert et al., 2014; Danbolt, 2001). The uptake of Glu from extracellular fluids is mainly mediated by glutamate/aspartate transporter (GLAST/EAAT1) and glutamate transporter 1 (GLT-1/EAAT2), which are primarily expressed by astrocytes (Foran and Trotti, 2009). Excitatory amino acid carrier 1 (EAAC1/EAAT3) is widely expressed in neurons (Holmseth et al., 2012) and has high affinity with Cys. In particular, EAAC1 preferentially transports Cys, rather

Abbreviations: TCMs, traditional Chinese medicines; BBB, blood-brain barrier; ORT, object recognition task; γ-GCS, γ-glutamylcysteine synthetase; EAATs, excitatory amino acid transporters; MRP-1, multidrug resistance-associated protein 1; γ-GT, γ-glutamyl transpeptidase; CMC-Na, sodium carboxymethylcellulose; MD, microdialysis; HG-FAAS, hydride generation flame atomic absorption spectrometry; HPLC, high-performance liquid chromatography.

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than Glu, into neurons (Chen and Swanson, 2003). Intriguingly, astrocytes contain high reserve levels of GSH and can export intracellular GSH through multidrug resistance-associated protein 1 (MRP-1) (Dringen and Hirrlinger, 2003). The released GSH is then cleaved into Cys and Gly via γ-glutamyl transpeptidase (γ-GT) and aminopeptidase N (AP-N). Cys is subsequently transported into neurons by EAAC1 for GSH synthesis. System X_C^- contains a light chain named xCT and acts to specifically transport extracellular Cys_2 and intracellular Glu (Lewerenz et al., 2013). After uptake by system X_C^-, Cys_2 is rapidly reduced to Cys for GSH synthesis. These data indicate that the main determining factors in neuronal GSH synthesis involve the transport of precursors by GLAST, GLT-1, EAAC1 and xCT, the precursor Cys provided by MRP-1and γ-GT, the rate-limiting enzyme γ-GCS and its regulatory factor Nrf2, and the precursor Glu in the extracellular fluid. However, whether the arsenic contained in realgar interferes with the neuronal synthesis of GSH by affecting the above-mentioned determining factors and the molecular mechanisms underlying these effects remain unknown. To address these deficits, in the present study, we used different doses of realgar to induce and analyze the neurotoxicity caused by realgar and the molecular mechanisms underlying its effects. These data provide a theoretical basis for preventing the drug-induced chronic arsenic poisoning of the nervous system that is triggered by realgar.

2. Materials and methods

2.1. Antibodies and reagents

Realgar (>90% As_4S_4) was obtained from Gannan Tibetan Autonomous Prefecture Medicine Company (Gansu, China). Na_2S standard (>98%, $Na_2S \cdot 9H_2O$) was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Glu, GSH, Cys standards, dansyl chloride and monobromobimane (MBB) were obtained from Sigma Chemical Corporation (St. Louis, MO, USA). The standard reference material for sodium arsenite ($NaAsO_2$) was obtained from Fluka Chemical Corporation (USA). Primary rabbit antibodies that recognize GLAST (ab416), GLT-1 (ab178401), EAAC1 (ab124802), HO-1 (ab13243), GCLC (ab19068), GCLM (ab153967) and mouse anti-NSE (ab16808) antibodies were obtained from Abcam (Cambridge, MA, USA). Rabbit anti-Nrf2 (sc-13032), xCT (sc-98552), actin (sc-7210), and mouse anti-MRP-1 (sc-53130) antibodies and the horseradish peroxidase (HRP)-conjugated goat anti rabbit/mouse secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TRITC/FITC–tagged anti-rabbit/mouse secondary antibodies were purchased from Zhongshan Bioengineering Institute (Beijing, China). The enhanced chemiluminescence (ECL) plus kit was obtained from Amersham Life Science (Buckinghamshire, UK). TRIzol reagent and real-time polymerase chain reaction (real-time PCR) kits were purchased from Takara Biotechnology (Dalian, China). The ELISA analysis kits used to analyze γ-GT were provided by R&D System Inc. (Minneapolis, USA). Sodium borohydride and sodium hydroxide were purchased from Wako Pure Chemical Industries Ltd. (Japan). All other chemicals were obtained from local chemical suppliers. All chemicals were of analytical grade, high-performance liquid chromatography (HPLC) grade or the best pharmaceutical grade available.

2.2. Animals and treatments

One hundred and fifty-two male Institute of Cancer Research (ICR) mice with initial body weights of 18–22 g were obtained from the Experimental Animal Center at China Medical University. Mice were housed at five mice per cage under standard laboratory conditions including 24 ± 1 °C with a relative humidity of 50 ± 5% and a 12 h light/dark cycle. Mice were acclimatized to laboratory conditions prior to the experiment. All mice were randomly divided into four groups by weight. There were 38 mice in each group: the control group and realgar-treated groups (0.15 g/kg, 0.45 g/kg and 1.35 g/kg). The control group was intragastrically (i.g.) treated with 0.5% (w/v) sodium carboxymethylcellulose (CMC-Na), whereas the realgar-treated groups were exposed to realgar suspended in CMC-Na daily for eight consecutive weeks at concentrations of 0.15 g/kg, 0.45 g/kg or 1.35 g/kg body weight. During the experiment, all mice were allowed free access to food and water, and the mice were observed for symptoms of toxicity every day.

Twenty mice from each group were submitted to object recognition task (ORT) testing. After the ORT tests were performed, eight mice underwent microdialysis (MD) to determine extracellular Glu and Cys levels. After MD, these mice were anesthetized and sacrificed by decapitation prior to the removal of the hippocampus, which was used to determine GSH levels. The tissues obtained from four mice in each group were used in immunofluorescence experiments to measure the protein expression of xCT, EAAC1, MRP-1 and Nrf2. Eight mice from each group were anesthetized and sacrificed, and the hippocampus was subsequently removed to measure the mRNA and protein expression levels of GLAST, GLT-1, EAAC1, xCT, Nrf2, HO-1, MRP-1, GCLC and GCLM. The tissues of two mice from each group were used to analyze the ultrastructural features of hippocampal neurons and synapses. The hippocampi of eight mice from each group were used to determine γ-GT levels. The last eight mice in each group were anesthetized, and whole blood samples were collected from the peripheral vessels to determine arsenic levels. The brains of these animals were quickly removed to measure the levels of arsenic and active sulfur.

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Shenyang, Liaoning, China). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Care was taken to avoid causing suffering and to minimize the number of animals used.

2.3. Determination of arsenic levels in the blood and brain

The levels of arsenic in the blood and brain were determined according to the protocols described in previous reports (Huo et al., 2012). Briefly, brain samples (100 mg) or blood (0.5 mL) was homogenized in 1 mL of deionized water, which was then mixed with 1 mL of 3 M H_2SO_4. The samples were digested in a focused microwave field for 10 min. The digested samples were assayed for arsenic levels using hydride generation flame atomic absorption spectrometry (HG-FAAS).

2.4. ORT tests

ORT tests were performed six days before the termination of realgar exposure according to the methods of Heyser and Ferris (2013), with some modifications. The test comprised three successive trials. The first trial consisted of placing the mouse in the center of an empty open field box (60 cm length × 60 cm width × 60 cm height) and allowing the mouse to freely explore the box for 3 min (i.e., the habituation phase). After this, the mice were returned to their home cage. The second trial commenced the next day and involved placing the mouse in the center of the same box, which now had two identical objects that were placed on opposite sides of the box, and allowing the mouse to freely explore the objects for 3 min (i.e., the training phase). After 3 days of training, the animals were placed back in their cages and allowed to rest for 1 day. The third trial was performed on the last day. Each mouse was placed back into the same box, wherein one of the objects used during training was replaced by a novel object. Each mouse was then allowed to freely explore for 3 min (i.e., the testing phase). All objects presented similar textures, colors, and sizes but had distinctive shapes. Exploration of an object was defined as orienting toward the object with the animal's nose within 1 cm of the object and/or touching it with the nose. Exploratory preference, which was calculated as the ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used as the measurement of recognition memory.