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PII: S0006-8993(14)01753-3
DOI: http://dx.doi.org/10.1016/j.brainres.2014.12.046
Reference: BRES44013

To appear in: Brain Research

Accepted date: 10 December 2014

Cite this article as: Ying Guo, Hua Yuan, Lulu Jiang, Junlin Yang, Tao Zeng, Keqin Xie, Cuili Zhang, Xiulan Zhao, Involvement of decreased Neuroglobin protein level in cognitive dysfunction induced by 1-bromopropane in rats, Brain Research, http://dx.doi.org/10.1016/j.brainres.2014.12.046

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Involvement of decreased neuroglobin protein level in cognitive dysfunction induced by 1-bromopropane in rats

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ABSTRACT

1-Bromopropane (1-BP) is used as a substitute for ozone-depleting solvents (ODS) in industrial applications. 1-BP could display central nervous system (CNS) neurotoxicity manifested by cognitive dysfunction. Neuroglobin (Ngb) is an endogenous neuroprotectant and is predominantly expressed in the nervous system. The present study aimed to investigate Ngb involvement in CNS neurotoxicity induced by 1-BP in rats. Male Wistar rats were randomly divided into 5 groups (n=14) and treated with 0, 100, 200, 400 and 800 mg/kg bw 1-BP, respectively, by gavage for consecutive 12 days. Rats displayed cognitive dysfunction dose-dependently through Morris water maze (MWM) test. Significant neuron loss in layer 5 of the prelimbic cortex (PL) was observed. Moreover, 1-BP decreased Ngb protein level in cerebral cortex and Ngb decrease was significantly positively correlated with cognitive dysfunction. Glutathione (GSH) content, GSH/oxidized glutathione (GSSG) ratio and glutamate cysteine ligase (GCL) activity decreased in cerebral cortex, coupled with the increase in GSSG content. GSH and GSH/GSSG ratio decrease were significantly positively correlated with cortical Ngb decrease. Additionally, levels of N-epsilon-hexanoyl-lysine (HEL) and 4-hydroxy-2-nonenal (4-HNE) modified proteins in cerebral cortex of 1-BP-treated rats increased significantly. In conclusion, it was suggested that 1-BP resulted in decreased endogenous neuroprotectant Ngb in cerebral cortex, which might play an important role in CNS neurotoxicity induced by 1-BP and that 1-BP-induced oxidative stress in cerebral cortex might partly be responsible for Ngb decrease.
Key words: 1-Bromopropane; Neurotoxicity; Oxidative stress; Neuroglobin

1. Introduction

1-Bromopropane (1-BP), regarded as a highly volatile organic compound, has a transient existence with a half-life of approximately 15 days in the ambient environment (Boublík et al., 1984; Nelson et al., 1997). Additionally, compared to chlorofluorocarbons (CFCs) and hydrochlorofluorocarbons (HCFCs), 1-BP has a lower ozone depleting potential (ODP) of 0.013-0.018 at middle latitude (USEPA). Therefore, as an alternative to ozone-depleting solvents (ODS), 1-BP is widely applied in industrial uses, such as cleaning metals and electronic components (OSHA/NIOSH) and manufacturing of pesticides, pharmaceuticals, flavors and fragrances (NTP). However, 1-BP could induce sensory and motor dysfunction, and central nervous system (CNS) disadvantages including reduced short-term memory in

Abbreviations: 1-BP, 1-bromopropane; 4-HNE, 4-hydroxy-2-nonenal; AD, Alzheimer’s disease; CFCs, chlorofluorocarbons; HCFCs, hydrochlorofluorocarbons; HEL, N-epsilon-hexanoyl-lysine; ODP, ozone depleting potential; ODS, ozone-depleting solvents; CNS, central nervous system; PNS, peripheral nervous system; PL, prelimbic cortex; PUFA, polyunsaturated fatty acid; Mrps, multidrug resistance proteins; MWM, Morris water maze; GCL, glutamate cysteine ligase; GR, glutathione reductase; GS, glutathione synthetase; GSH, glutathione; GSSG, oxidized glutathione; ROS, reactive oxygen species; Ngb, neuroglobin; PFA, paraformaldehyde; PBS, phosphate buffer saline; BSA, Bovine Serum Albumin; DBA, diaminobenzidine
humans (Ichihara et al., 2002; Ichihara et al., 2004; Majersik et al., 2007; Sclar, 1999). In animal experiments, rats began to show peripheral nervous system (PNS) deficits after 4 weeks of exposure to 800 ppm 1-BP; whereas abnormal CNS signs were observed in rats exposed to 50 or 200 ppm 1-BP for only 3 weeks (Honma et al., 2003; Ichihara et al., 2000). Based on previous studies, 1-BP was concluded to induce CNS and PNS neurotoxicity, the former occurring earlier than the latter. Therefore, researches on the dose-dependent severity of 1-BP-induced CNS neurotoxicity and its possible mechanisms might be beneficial to early identification, prevention and treatment of 1-BP intoxication. Furthermore, cognitive dysfunction, contained in chief complaints of most human cases and abnormal CNS signs of intoxicated animals, could be regarded as an indicator of CNS neurotoxicity of 1-BP and reliably quantified by Morris Water Maze (MWM) test in animal researches (Vorhees and Williams, 2006).

Cognitive performances in MWM is associated with several brain regions such as cerebral cortex, hippocampus, basal forebrain, striatum and cerebellum (D’Hooge and De Deyn, 2001). Previous studies proved that lesions in cerebral cortex might be relevant to cognitive dysfunction and that MWM could be considered as one of the measures of cortical function (Brown et al., 2000; D’Hooge and De Deyn, 2001; Rinwa and Kumar, 2012; Scheff et al., 1997). Hence, this study discussed cortical changes induced by 1-BP.

Based on many previous researches on cognitive disorders, oxidative stress was proposed to have a possible causal relationship with cognitive dysfunction (Andersen,
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2004; Chang et al., 2011; Yan et al., 2013). In CNS, glutathione (GSH) is the primary intracellular antioxidant and plays an important role in detoxification of reactive oxygen species (ROS) and xenobiotics. Based on previous studies, it was indicated that stable GSH content was essential for maintaining normal cognitive function and that GSH depletion might partly contribute to cognitive dysfunction, although the causation was often suggested (An et al., 2012; Ballatori et al., 2009; Dean et al., 2009; Liu et al., 2004; Martin and Teismann, 2009; Shukitt-Hale et al., 1998; Yabuki and Fukunaga, 2013). Recent studies assumed that 1-BP-induced CNS neurotoxicity was associated with oxidative stress and GSH depletion (Huang et al., 2011; Huang et al., 2012; Subramanian et al., 2012). However, the mechanism of oxidative stress and GSH depletion induced by 1-BP has not been fully illustrated yet.

In addition to GSH, there are other important antioxidants present in the nervous system among which neuroglobin is a novel antioxidant and plays neuroprotective roles. Ngb, firstly discovered in 2000, is expressed in neurons, astrocytes, retina and some endocrine tissues (Burmester et al., 2000; Burmester and Hankeln, 2004; De Marinis et al., 2013a). Brunori and Vallone (2007) demonstrated its highly conserved structure and function. As the firstly discovered hexacoordinate hemoglobin in vertebrates, Ngb has a heme iron, either in ferrous (reduced) or ferric (oxidized) form. Ngb was generally accepted to have neuroprotective effects, due to the evidences that overexpression of Ngb protected neural cells from hypoxic-ischemia injury (Khan et al., 2006; Sun et al., 2001), oxidative stress-induced cell death (Fordel et al., 2006; Liu et al., 2009) and beta-amyloid neurotoxicity (Khan et al., 2007; Li et al., 2008b) in
vitro, and protected animals from experimental stroke (Sun et al., 2003) and Alzheimer’s disease (AD) (Khan et al., 2007) in vivo. Although not fully elucidated, there are several possible mechanisms of the neuroprotective roles of Ngb, involving reactive species scavenging (Yu et al., 2009) and anti-apoptosis (Brittain, 2012) in the processes of which Ngb works in a ferrous state and then turns to ferric Ngb which could be converted to ferrous Ngb again by utilization of the intracellular antioxidants (Hota et al., 2012). Cognitive dysfunction is associated with ROS-induced oxidative damage and unexpected apoptosis of neural cells in the cerebral cortex the severity of which, to some degree, is probably connected with the level of Ngb expression and the conversion of ferric Ngb to ferrous Ngb.

On account of the neuroprotective effects of Ngb, this study aimed to investigate Ngb involvement in CNS neurotoxicity induced by 1-BP in rats. The exposure levels of 1-BP were selected based on our preliminary experiment as well as our previous experiment estimating 1-BP PNS toxicity (Wang et al., 2012), aiming to mimic actual clinical manifestations of human 1-BP-intoxicated cases. In our previous experiment, exposure to 400 and 800 mg/kg bw 1-BP for 16 weeks could induce significant decrease in hindlimb grip strength and paralysis which were similar to clinical signs of human cases. In the early weeks in that experiment, we also observed 1-BP-induced CNS toxic effects, such as emotional changes. Furthermore, in our preliminary experiment, it was indicated that 200, 400 and 800 mg/kg bw 1-BP could induce CNS neurotoxicity manifested by cognitive dysfunction which was similar to clinical symptoms of human cases. Therefore, 100, 200, 400 and 800 mg/kg bw 1-BP
were used as intoxication doses. Neuron loss in prelimbic cortex (PL) was estimated and Ngb protein level in cerebral cortex was measured. Since the redox state was critical to the conversion of ferric Ngb to ferrous Ngb and the protein level of Ngb, GSH content and lipid peroxidation condition in cerebral cortex were determined. Besides, glutamate cysteine ligase (GCL) activity was measured to investigate the possible mechanism of GSH alteration. Ngb involvement in CNS neurotoxicity induced by 1-BP has not been discussed by previous studies. Therefore the data in the current study might provide further understanding of 1-BP-induced CNS neurotoxicity and its underlying mechanisms.

2. Results

2.1. Alterations in body weight of 1-BP-treated rats

A two-way repeated-measures ANOVA of body weight revealed significant effects of exposure ($F_{(2, 91)}=194.239, p<0.001$), dose ($F_{(4, 45)}=5.806, p=0.001$), and the interaction of exposure $\times$ dose ($F_{(8, 91)}=5.497, p<0.001$) on the body weight gain of rats. Post hoc multiple comparisons showed that rats treated with 800 mg/kg bw 1-BP displayed significant loss of body weight gain ($p<0.05$) over the experimental days, compared to control group. Subsequent one-way ANOVA indicated that there was no difference in the initial body weight among all groups and that rats exposed to 800 mg/kg bw 1-BP exhibited significant decrease in body weight gain on day 4, 8, 12 of the experiment ($p<0.05$, $p<0.01$), compared to control group (Figure 1). No death of rats was recorded during the treatment period.
2.2 1-BP-treated rats displayed cognitive dysfunction in MWM test

In the spatial navigation test, escape latency and distance travelled were analyzed using two-way repeated-measures ANOVA. Dose acted as the between-subjects factor and exposure acted as the within-subjects factor. As shown in Figure 2A, a significant effect of exposure ($F_{(2, 101)} = 73.916, p<0.001$) on escape latency was observed, indicating that performances of all groups improved across days. Furthermore, there was a significant effect of exposure $\times$ dose interaction ($F_{(9, 101)} = 2.724, p=0.007$), indicating that the effect of exposure varied for different doses of 1-BP. In addition, a significant effect of dose ($F_{(4, 45)} = 27.016, p<0.001$) was observed, indicating that 1-BP induced a dose-dependent increase in escape latency. Bonferroni’s test revealed that rats treated with 400 and 800 mg/kg bw 1-BP showed significantly increased escape latencies ($p<0.01$), compared to control group. Subsequent one-way ANOVA indicated that rats exposed to 400 and 800 mg/kg bw 1-BP began to display remarkably increased escape latencies from the first training day (8th experimental day) ($p<0.01, p<0.05$), compared to control group. Similar result was drawn from the analysis of distance travelled (exposure: $F_{(3, 135)} = 63.262, p<0.001$; dose: $F_{(4, 45)} = 23.973, p<0.001$; and exposure $\times$ dose interaction: $F_{(12, 135)} = 3.308, p<0.001$). Bonferroni’s test suggested significant increase in distance travelled for 400 and 800 mg/kg bw 1-BP group ($p<0.05$ and $p<0.01$ respectively), compared to control group. Subsequent one-way ANOVA showed that, compared to control group, rats exposed to 400 and 800 mg/kg bw 1-BP started to exhibit noticeably incremental distance travelled from the first training day (8th experimental
day) \((p<0.01, p<0.05)\) (Figure 2B). Swimming speed was used to estimate the motoric activity of rats in MWM test. As indicated in Figure 2C, no significant difference in swimming speed was generally found between exposures \((F_{(3,135)}=1.622, p=0.187)\), between doses \((F_{(4,45)}=1.944, p=0.119)\) and between interactions of exposure \(\times\) dose \((F_{(12,135)}=0.197, p=0.998)\). Thus, performance deficits of 1-BP-treated rats in spatial navigation test were not likely due to a locomotor effect. According to the results above described, it was suggested that 1-BP impaired spatial learning ability of rats dose-dependently.

In the spatial probe trial, spatial memory was evaluated by number of platform crossing and percentage of time spent in target quadrant. There were significant decrease in number of platform crossing in both 400 and 800 mg/kg bw 1-BP group \((p<0.01)\), compared to control group (Figure 3A). Figure 3B indicated a negative, dose-dependent effect of 1-BP on percentage of time spent in target quadrant and 200, 400, 800 mg/kg bw 1-BP group showed significant difference with control group \((p<0.05, p<0.01)\). No significant difference in swimming speed was generally found between groups (Figure 3C), suggesting no contribution of locomotor effect to performance deficits in spatial probe trial either. Therefore, it was indicated that 1-BP induced spatial memory impairment of rats dose-dependently.

2.3 1-BP induced significant neuron loss in layer 5 in PL

As a part of prefrontal cortex, PL has an important role in cognitive function. Therefore, the numbers of neurons in different layers of PL were estimated to evaluate
the effect of 1-BP on PL. As indicated in Figure 4, 1-BP induced significant and dose-dependent decrease in neuron population in layer 5 of PL. However, there was no significant changes in neuron counting in layer 2/3 and layer 6 induced by 1-BP.

2.4 1-BP decreased Ngb protein level in cerebral cortex and Ngb decrease was significantly positively correlated with cognitive dysfunction

As indicated in Figure 5A, 1-BP displayed a negative, dose-dependent effect on Ngb protein level and compared to control group, Ngb protein levels of 200, 400, 800 mg/kg bw group decreased significantly ($p<0.01$).

To assess the relationship between Ngb protein level and spatial learning ability, correlation analyses were performed between Ngb protein level and escape latency or distance travelled on the 4th training day which might be most similar to the potentially actual performances corresponding to the measured Ngb. It was demonstrated that Ngb protein level showed significantly negative correlations with escape latency (Figure 5B, $r=-1$, $p<0.01$) and distance travelled (Figure 5C, $r=-1$, $p<0.01$), and positive correlations with percentage of time spent in target quadrant (Figure 5D, $r=1$, $p<0.01$) and number of platform crossing (Figure 5F, $r=0.975$, $p<0.01$), indicating that Ngb decrease was significantly positively correlated with cognitive dysfunction.

2.5 1-BP induced reduction in GSH content and GSH/GSSG ratio, increment in GSSG content and suppression of GCL activity in cerebral cortex

GSH content and GSH/ oxidized glutathione (GSSG) ratio represent the redox
status, which are crucial for maintaining cellular redox homeostasis. As shown in Figure 6A, compared to control group, there was dose-dependent decrease in GSH content in cerebral cortex, statistically significant for all 1-BP treatment groups ($p<0.01$), suggesting that 1-BP induced considerable oxidative stress even at a low dose of 100 mg/kg bw. The GSH content of 800 mg/kg bw group decreased to 55% of the control value. Significant increase in GSSG content in cerebral cortex were observed in 1-BP treatment groups ($p<0.01$), compared to the control group (Figure 6B). Furthermore, GSH/GSSG ratio was calculated and Figure 6C indicated that 1-BP dose-dependently decreased GSH/GSSH ratio in cerebral cortex ($p<0.01$) with the lowest ratio being 46% of the control value in 800 mg/kg bw group. Results described above suggested that 1-BP disturbed redox homeostasis.

To discuss the possible mechanism of GSH decrease, the activity of GCL which was responsible for GSH de novo synthesis was evaluated. As indicated in Figure 6D, compared to control group, 1-BP treatment significantly suppressed GCL activity ($p<0.01$), suggesting inhibition of GSH de novo synthesis.

2.6 GSH decrease and GSH/GSSG ratio reduction in cerebral cortex were significantly positively correlated with cortical Ngb decrease and cognitive dysfunction

Figure 7A and Figure 7B showed that Ngb protein level was significantly positively correlated with GSH content ($r=0.9, p<0.05$) and GSH/GSSG ratio ($r=0.9, p<0.05$) in cerebral cortex, suggesting that decrease in Ngb protein level might be associated with decreased GSH pool.
Correlations of cognitive performances in MWM test with GSH content and GSH/GSSG ratio of cerebral cortex were assessed. GSH content of cerebral cortex showed significantly negative correlations with escape latency (r=-0.736, \( p<0.01 \); Figure 8A) and distance travelled (r=-0.758, \( p<0.01 \); Figure 8B), as well as positive correlations with percentage of time spent in target quadrant (r=0.570, \( p<0.01 \); Figure 8C) and number of platform crossing (r=0.378, \( p<0.01 \); Figure 8D), indicating positive correlation between GSH decrease and cognitive dysfunction induced by 1-BP. Similar results were drawn from correlation analyses between GSH/GSSG ratio and cognitive performances. GSH/GSSG ratio of cerebral cortex was significantly negatively correlated with escape latency (r=-0.745, \( p<0.01 \); Figure 8E) and distance travelled (r=-0.737, \( p<0.01 \); Figure 8F), and positively with percentage of time spent in target quadrant (r=0.581, \( p<0.01 \); Figure 8G) and number of platform crossing (r=0.419, \( p<0.01 \); Figure 8H), suggesting that there was positive correlation between GSH/GSSG ratio reduction and cognitive deficits induced by 1-BP.

2.7 1-BP induced lipid peroxidation in cerebral cortex

To evaluate the extent of lipid peroxidation induced by 1-BP, levels of N-epsilon-hexanoyl-lysine (HEL) and 4-hydroxy-2-nonenal (4-HNE) modified proteins were quantified. There were significant increase in HEL modified proteins in rats treated with 200, 400 and 800 mg/kg bw 1-BP, compared to rats in control group (\( p<0.01 \)) (Figure 9A). As shown in Figure 9B, levels of 4-HNE modified proteins in 1-BP treatment groups were significantly higher than that in control group (\( p<0.01 \)).
was indicated that 1-BP induced significant lipid peroxidation in cerebral cortex in rats even at a low dose of 100 mg/kg bw.

3. Discussion

The present study indicated that significant CNS neurotoxicity was observed as early as 1 week after starting the consecutive exposure to 400 and 800 mg/kg bw 1-BP and 10 days after starting the exposure to 200 mg/kg bw 1-BP. Although rats exposed to 100 mg/kg bw 1-BP did not exhibit significant learning and memory disadvantages, cognitive function deteriorated dose-dependently.

1-BP could induce neuropathologic changes in PL, one part of the prefrontal cortex. It was indicated that 1-BP resulted in significant and dose-dependent neuron loss in layer 5 of PL. However, there was no significant changes in neuron counting in layer 2/3 and layer 6 induced by 1-BP. Prefrontal cortex has important roles in working memory and memory storage (Runyan et al., 2004). Both humans and animals with prefrontal cortex damage could display memory deficits (Markowitsch et al., 1993; Runyan et al., 2004). Prelimbic cortex has essential roles in CNS projections. It receives extensive projections from limbic regions and it also projects back to limbic cortical regions, which are important in working memory and cognition (Paxinos, 2004). Previous studies demonstrated that working memory deficits were always coupled with morphological changes and dendrite abnormalities within layer 5/6 neurons of the PL (Hoskison et al., 2009). In the present study, significant neuron loss in layer 5 of the PL was observed, suggesting
that 1-BP could induce adverse effects on cortical neurons dose-dependently in vivo and that neuron loss in layer 5 of the PL might have a role in 1-BP-induced cognitive dysfunction.

CNS neurotoxicity of 1-BP was assumed to be associated with oxidative stress in different regions of brain, including cerebral cortex (Zhong et al., 2013), hippocampus (Huang et al., 2012) and cerebellum (Subramanian et al., 2012). When confronting oxidative stress, in addition to the main antioxidant GSH, there are other protection mechanisms such as the neuroprotectant Ngb. Ngb could be operating as an endogenous scavenger for reactive species to attenuate oxidative stress (Yu et al., 2009). It was suggested that Ngb was able to scavenge NO, \( \text{H}_2\text{O}_2 \), peroxynitrite and superoxide (Herold et al., 2004; Yamashita et al., 2014). Oxygenated Ngb (Ngb-Fe\(^{2+}\)-O\(_2\)), formed by the autooxidation of Ngb, could react with NO rapidly to produce NO\(^{3-}\) and met-Ngb (Ngb-Fe\(^{3+}\)) (Brunori et al., 2005). Accumulated data also suggested that overexpression of Ngb could ameliorate \( \text{H}_2\text{O}_2 \)-mediated and \( \beta \)-amyloid-induced oxidative damage to cultured cells (Fordel et al., 2006; Li et al., 2008a; Li et al., 2008b). Moreover, Ngb could play a functional role against apoptosis of neural cells (Brittain et al., 2010; Brittain, 2012) which could be triggered by oxidative stress (Circu and Aw, 2008; Circu and Aw, 2010; Franco and Cidlowski, 2009) through mitochondrial pathway (Orrenius et al., 2007; Ott et al., 2007). Ngb protein level was supposed to determine the threshold level of mitochondrial cytochrome c release essential for triggering apoptotic cell death (Fago et al., 2008). Lan et al. (2014) and De Marinis et al. (2013b) reported that Ngb overexpression...
decreased neuronal apoptosis *in vivo and in vitro*. A quantitative model of apoptosis inhibition by Ngb also suggested that Ngb exhibited its neuroprotection concentration-sensitively (Raychaudhuri et al., 2010). The current study demonstrated that 1-BP treatment decreased Ngb protein level in cerebral cortex in a dose-dependent manner. Ngb was expressed in neurons as well as astrocytes (De Marinis, 2013). Therefore, Ngb decrease induced by 1-BP made the cortical neurons more vulnerable to damage by reactive species and apoptosis which could cause neuron loss. Furthermore, previous researches on cognitive disorders such as AD indicated that decreased level of Ngb was observed to be coupled with cognitive dysfunction in animals (Chen et al., 2012) and that transgenic overexpression of Ngb was beneficial to memory performance (Khan et al., 2007), suggesting a protective role of Ngb in cognitive function which was one aspect of its neuroprotective effects. This study demonstrated that Ngb decrease was significantly positively correlated with cognitive dysfunction induced by 1-BP, indicating that Ngb decrease might play an important role in 1-BP-induced cognitive dysfunction and CNS neurotoxicity.

The neuroprotective roles of Ngb demand a reducing cellular environment, allowing the reduction of ferric state to ferrous state to maintain the balance between reduced and oxidized Ngb. When oxidative stress was induced and the redox homeostasis was destroyed, ferric Ngb would be accumulated, ubiquitinated and then decomposed, resulting in excessive Ngb decrease (Hota et al., 2012). To investigate the possible mechanism of Ngb decrease in cerebral cortex induced by 1-BP, contents of GSH and GSSG and lipid peroxidation condition were determined. It was
demonstrated that rats of all 1-BP treatment groups exhibited significant reduction of GSH content and GSH/GSSG ratio, coupled with increment in GSSG content, in cerebral cortex even at a relatively low dose of 100 mg/kg bw where there was no significant behavioral changes. Previous studies demonstrated that depleted GSH could result in memory deficits (Ansari et al., 2008; Zhang et al., 2014). Correlation analyses in the present study demonstrated that cognitive function was closely related to GSH content and GSH/GSSG ratio. However, how much contribution Ngb has made to these relationships needs further researches. Lipid peroxidation condition was manifested by the levels of HEL and 4-HNE modified proteins. HEL is considered as a good biomarker of the initial stage of lipid peroxidation in that it is an early product of its process (Kato and Osawa, 2010). HEL is generated from the assault of 13-hydroperoxyoctadienoic acid (13-HPODE), a lipid hydroperoxide produced from omega-6 unsaturated fatty acid, to lysine residues in proteins (Kato et al., 2004). 4-HNE, as a predominant end-product, is recognized as an indicator of the later phase of lipid peroxidation (Uchida et al., 1999). The current results demonstrated the elevated levels of HEL and 4-HNE modified proteins in cerebral cortex induced by 1-BP. Therefore, it was suggested that 1-BP-induced oxidative stress could result in an oxidizing environment and might partly be responsible for Ngb decrease, in accord with the results of correlation analyses that decrease in Ngb protein level was closely positively correlated with reduction in GSH content and GSH/GSSG ratio in cerebral cortex.

1-BP-induced oxidative stress was considered to start with the oxidation of 1-BP
to form 1-bromo-2-propanol and bromoacetone both of which, as well as 1-BP itself, could react with GSH to generate different conjugates to consume GSH (Garner et al., 2006). The supplement of GSH mainly depends on de novo synthesis and reduction of GSSG to GSH in CNS. The de novo synthesis of GSH requires two sequential reactions catalyzed by GCL and GSH synthetase (GS), respectively. GCL is the rate-limiting enzyme in GSH synthesis (Lu, 2013). GSH is also generated through the reduction of GSSG by cellular glutathione reductase (GR) (Schirmer RH, 1989). Our laboratory (Zhong et al., 2013) previously reported that 1-BP significantly decreased GR activity in cerebral cortex of rats, indicating slow reduction of GSSG to GSH and accumulation of GSSG. Accumulated GSSG could be exported from the cells by multidrug resistance proteins (Mrps) and the exported GSSG was considered no longer to act as substrate for cytoplasmic GSH reproduction by GR (Schmidt and Dringen, 2012). GCL activity was crucial for compensation for GSH consumption and the effect of 1-BP on GCL activity has not been discussed by previous studies. Thereby, GCL activity in cerebral cortex was determined. The present study showed that GCL activity in cerebral cortex was also significantly suppressed by 1-BP, resulting in inadequate GSH de novo synthesis and insufficient compensation for GSH loss in neural cells. Decreased GSH level would result in the overproduction of ROS which could target on polyunsaturated fatty acid (PUFA) to trigger lipid peroxidation. Neurotoxic agents produced during lipid peroxidation would consume GSH in return to form a vicious cycle, thus amplifying oxidative stress.

1-BP induces CNS as well as PNS neurotoxicity in workers and the occupational
exposure was based primarily on inhalation route at workplace. Generally, the administration route in animal experiments should be related to anticipated human exposure. However, as one of the most convenient and accurate route of administration, gavage has also been applied in the researches of 1-BP and other volatile neurotoxicants. The rationality of gavage has been discussed in previous studies (Zhong et al., 2013). The rats exposed to 1-BP by gavage exhibited similar neurotoxic symptoms observed in human cases. In this regard, the findings may be useful for pathogenesis study of neurologic disadvantages caused by 1-BP in humans and provide data on its oral toxicity. Admittedly, there may be differences between the oral and inhalation neurotoxicity of 1-BP, which may be revealed by the comparison of pharmacokinetic processes.

In summary, the present study demonstrated that 1-BP intoxication led to cognitive dysfunction and neuron loss in layer 5 of the PL. 1-BP induced Ngb decrease in cerebral cortex. Meanwhile, 1-BP induced oxidative stress in cerebral cortex manifested by GSH depletion and elevated levels of HEL and 4-HNE modified proteins. These data suggested that decreased endogenous neuroprotectant Ngb in cerebral cortex might play an important role in CNS neurotoxicity induced by 1-BP and 1-BP-induced oxidative stress in cerebral cortex might partly be responsible for Ngb decrease. However, whether Ngb could be directly depleted by 1-BP and its reactive metabolites needs more researches.
4. Experimental procedure

4.1 Materials

1-BP (99.99%) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The detection kits for GSH and GSSG content were obtained from Beyotime Institute of Biotechnology (Haimen, China). The detection kit for GCL activity was purchased from Suzhou Comin Biotechnology Co., Ltd (Suzhou, China). Anti-HEL monoclonal antibody (clone 5F12) and anti-4-HNE monoclonal antibody (clone HNEJ-2) were provided by JaICA (NOF Co., Tokyo, Japan). Anti-Ngb polyclonal antibody and anti-β-actin monoclonal antibody (clone AC-15) were obtained from Sigma (St. Louis, MO, USA). Anti-NeuN antibody ([EFR12763]; Neuronal Marker ab177487) was purchased from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated IgG and biotinylated IgG were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Vestastain ABC kit was supplied from Vector Laboratories (Burlingame, CA). BCA™ Protein assay kit was bought from Pierce Biotechnology, Inc. (Rockford, IL, USA). Chemiluminescence detection kit for HRP was purchased from Biological Industries Israel Beit Haemek LTD (Kibbutz Beit Haemek, Israel). All other chemicals used were of the highest grade commercially available.

4.2 Ethics statement

The procedures and protocols of the study were approved by the Ethics Committee for Animal Experiments of Shandong University Institute of Preventive
Medicine ( Permit Number: 20111231) in accord with the NIH Guide for Care and Use of Laboratory Animals.

4.3 Animal treatment

Male Wistar rats, weighing 180-220 g, were provided by Vital River Laboratories (Beijing, China). Rats were kept in plastic cages with free access to tap water and standard rat diet. They were raised under controlled conditions with a 12 h light/dark cycle, approximately 22±2 °C room temperature and a relative humidity of 50-60 %. After 5 days of acclimation, 70 rats were randomly divided into 5 groups (n=14 rats/group) and treated with 0, 100, 200, 400 and 800 mg/kg bw 1-BP (dissolved in corn oil), respectively, by gavage for consecutive 12 days. 10 rats per group carried out the MWM test from experimental day 8 to 12 and then were decapitated on day 13. The cerebral cortexes were rapidly dissected, frozen in liquid nitrogen and stored at -80 °C. 4 rats per group were used for pathological measurement. On experimental day 13, 4 rats per group were anesthetized with intraperitoneal injection of 30% ethyl carbamate and perfused intracardially with saline followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS). Brains were removed and stored in 4% PFA (4°C).
4.4 MWM test

4.4.1 Apparatus

The apparatus and protocols of MWM test were as previously described (Zhong et al., 2013). Briefly, a black circular pool, 180 cm in diameter and 60 cm high, was filled with opaque water (25±1 °C) and surrounded by an opaque curtain. With four fixed-position marks (N, E, S, W) on the pool wall above the water surface, the maze was divided into four quadrants of equal size, i.e. NE, SE, SW, NW. The platform, hidden approximately 1-2 cm below the water surface, was placed in the middle of one quadrant and remained unmoved during the spatial navigation test. Rat movement paths were recorded and analyzed using a computerized tracking system (Huaibei ZhengHua Biological Instrument Equipment Co., Ltd., Suixi, China) comprised of a camera and the video tracking software.

4.4.2 Spatial navigation test

The spatial navigation test consisted of four trials per day for each rat and lasted for four training days. Rats were released into water with their heads pointing towards the pool wall randomly at the four starting positions of N, E, S and W. They were required to escape from the water and arrive onto the platform hidden beneath the water surface. Without seeing the platform, the location of it was only related to the visual marks on the pool wall and thus finding it required spatial memory. The swim paths of rats were recorded through the camera and analyzed on the computer by the tracking software. Different parameters describing the spatial learning function could
be calculated, such as escape latency (time to locate the hidden platform), distance travelled (path length to reach the platform) and swimming speed. In one trial, rats were allowed to swim until they found the platform or for 120 s at the most. Rats that did not find the platform were gently guided onto it and their latencies were recorded as 120 s. All rats were given a break of 30 s on the platform between trials.

4.4.3 Spatial probe trial

The spatial probe trial was performed on day 5 of the MWM test. The hidden platform was removed from the pool and the rats were released into water to swim for 90 s. To evaluate the spatial memory of rats, percentage of time spent in target quadrant in which the platform was previously located and number of platform crossing were calculated by the tracking software.

4.5 Neuron loss estimation

Brains were transferred to a solution of 30% sucrose for 3 days and then cut into series of 40-μm thick coronal sections on a freezing microtome. For each rat, in the same part of prefrontal cortex, every third section was selected and a set of 4 sections per rat was placed in 0.1 M PBS for the detection of NeuN, a neuronal marker. Free-floating sections were washed in PBS and then treated with 1% H₂O₂ for 15 min. Sections were then rinsed with PBS twice for 15 min each and blocked with 1% Bovine Serum Albumin (BSA), 4% normal goat serum and 0.4% TritonX-100 in PBS for 30 min at room temperature. Incubation with primary anti-NeuN antibody (1:500; ab177487; Abcam, Cambridge, UK) was performed with 5% BSA and 0.4% TritonX-100 in PBS at 4°C overnight. After removing the primary antibody, the
sections were rinsed with PBS twice for 15 min each and incubated with biotinylated secondary antibody in 0.3% Triton X-100 in PBS for 1 h at room temperature. Then the sections were rinsed with PBS twice for 15 min each and incubated in avidin/biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were then rinsed with PBS thrice for 10 min each and stained with a solution of 0.05% diaminobenzidine (DAB) and 0.03% H2O2. Afterwards, the sections were mounted on slides, air-dried, dehydrated in graded alcohol, cleared in xylene and coverslipped. Microscope was used to take photos and image analysis on sections was performed with Image J software (National Institutes of Health). Region and layer boundaries were determined according to the atlas of Paxinos and Watson (Paxinos and Watson, 2006) and clear cytoarchitectural differences (Paxinos, 2004). Prelimbic region (PL) of prefrontal cortex was selected to perform neuron counting due to that it had more discernible boundaries and that it is more clearly laminated. Strips of PL, 200 μm in width and in consistent location of every section, were collected and estimated. Neurons in each strip of PL were divided into three parts (layer 2/3, layer 5 and layer 6). Layer 1 is excluded from analysis due to its paucity of neuronal soma and rat PL does not have a layer 4. Number of neurons in each part was measured and recorded separately.

4.6 Determinations of GSH content, GSSG content and GCL activity in cerebral cortex of rats

4.6.1 Preparation of 10% homogenate of cerebral cortex

The protocol was as previously described (Zhong et al., 2013). In brief, 0.5 g of cerebral cortex of each rat was homogenized in 4.5 ml of ice-cold buffer comprising
0.01 M saccharose, 0.01 M Tris-HCl, 0.1 mM EDTA-Na$_2$ and 0.8% saline with a motor-driven glass homogenizer in an ice bath. The homogenates were kept in ice-water mixture for about 30 min and then centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were gathered, divided into aliquots and frozen at -80 °C for subsequent analysis. Protein concentrations of the supernatants were measured using BCA™ protein assay kits.

4.6.2 Determinations of GSH and GSSG contents

GSH and GSSG contents were measured by detection kits (Beyotime Institute of Biotechnology, Haimen, China) following the kit instruction. Briefly, for the determination of GSSG content, GSH in the supernatant of homogenate was eliminated and then GSSG remained was reduced to GSH by GR. The generated GSH could react with 5, 5’-dithiobis-(2-nitrobenzoic acid) (DTNB) to form yellow trinitrobenzene (TNB) which could be quantified spectrophotometrically at 412 nm. For the measurement of GSH content, total glutathione (GSH + GSSG) in the supernatant was determined by DTNB method without elimination of GSH in advance. The amount of GSH was obtained by subtracting GSSG content from total glutathione. The values were expressed as μM. The ratio of GSH to GSSG was calculated.

4.6.3 Determination of GCL activity

The activity of GCL was measured using commercial kit (Suzhou Comin Biotechnology Co., Ltd, Suzhou, China) following the kit instruction. With the existence of glutamate, cysteine, glycine, ATP and Mg$^{2+}$, GCL in the supernatant
could catalyze GSH synthesis at 37 °C, resulting in the dephosphorylation of ATP to generate inorganic phosphorus molecules. Through quantification of inorganic phosphorus molecules, GCL activity was calculated. Values were expressed as U/mg protein.

4.7 Western blotting analysis

Total protein extract was obtained described previously (Zeng et al., 2012). In brief, cerebral cortices were homogenized in 5 volumes of ice-cold RIPA lysis buffer (NaCl, 150 mM; leupeptin, 5 μg/ml; aprotinin, 5 μg/ml; EGTA, 1 mM; PMSF, 1 mM; NP-40, 0.5 %; sodium deoxycholate, 1 %; sodium dodecyl sulfate (SDS), 0.25 %; Tris-HCl, 50 mM, pH 7.4) with a motor-driven glass homogenizer in an ice bath. The homogenates were kept in ice-water mixture for approximately 30 min and then centrifuged at 20,800×g for 30 min at 4 °C. The supernatants were total protein extracts and the protein concentrations were quantified using BCA™ protein assay kits. The protein extracts were mixed with 3× loading buffer and then boiled for 10 min at 100 °C. The prepared protein aliquots (50 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5 % or 12 % gels), followed by transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The PVDF membranes were blocked with 5 % non-fat milk for 30 min at room temperature and then incubated with the primary antibodies overnight at 4 °C. After washing off unbound primary antibodies with Tris-buffered saline (TBS) containing 0.05 % Tween-20 (TBST), the membranes were incubated with HRP-conjugated
secondary antibody for 1 h at room temperature and then washed with TBST buffer again. Protein bands were detected by enhanced chemiluminescence system (ECL). Images were scanned and then the expression levels of proteins were quantified by densitometry analysis of specific protein bands, with normalization to β-actin, using Kodak Imaging Program and Image-Pro-Plus software (Eastman Kodak Company, New Haven, CT, USA).

4.8 Statistical analysis

All data were expressed as means ± SEM and analyzed statistically using SPSS 13.0 statistical software. Escape latency, distance travelled and swimming speed in spatial navigation test, as well as body weight, were analyzed by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni test, followed by one-way ANOVA coupled with LSD post hoc test for each training day. For all other data, one-way ANOVA was used with LSD post hoc test. Test of normality was conducted by Kolmogorov–Smirnov test. Correlation analyses were performed with Spearman correlation coefficient, except for that correlation analyses between spatial probe trial performances and GSH content and GSH/GSSG ratio were evaluated by Pearson’s correlation coefficient. A value of \( p < 0.05 \) was considered significant.

Conflict of interest

The authors declare no conflict of interest.
Author contributions

Conceived and designed the experiments: XZ, KX. Performed the experiments: YG, HY, LJ, JY. Analyzed the data: YG, TZ. Contributed reagents/materials/analysis tools: XZ, CZ. Wrote the paper: YG, XZ.

Acknowledgments

This research was supported by National Natural Science Foundation of China (81172708). We thank Ruirui Cou and Shasha Wang (Institute of Toxicology, School of Public Health, Shandong University, Jinan, China) for their assistance with western blot assays.

References


Chang, J., Siedlak, S., Moreira, P., Nunomura, A., Castellani, R.J., Smith, M.A., Zhu,


NTP, NTP technical report on the toxicology and carcinogenesis studies of 1-bromopropane (CAS No. 106-94-5) in F344/N rats and B6C3F1 mice


100, 3497-500.


Figure captions:

Figure 1. Effects of 1-bromopropane (1-BP) on the body weight of rats.

Significant loss of body weight gain was induced by exposure to 800 mg/kg bw 1-BP. Data were plotted as means ± SEM (n=10; *p<0.05, **p<0.01, vs. control group; two-way repeated-measures ANOVA with Bonferroni test followed by one-way ANOVA coupled with LSD post hoc test for each measuring day).

Figure 2. 1-bromopropane (1-BP)-treated rats displayed significant learning disadvantages in spatial navigation test in Morris Water Maze (MWM).

1-BP-treated rats displayed increased escape latency (A) and distance travelled (B). A locomotor effect was ruled out due to no significant difference in swimming speed (C). Data were graphed as means ± SEM (n=10; *p<0.05, **p<0.01, vs. control group; two-way repeated-measures ANOVA with Bonferroni test followed by one-way ANOVA coupled with LSD post hoc test for each training day).

Figure 3. 1-bromopropane (1-BP)-treated rats exhibited significant memory impairments in spatial probe trial in Morris Water Maze (MWM).

1-BP-treated rats exhibited decreased number of platform crossing (A) and percentage of time spent in target quadrant (B). A locomotor effect was ruled out due to no significant difference in swimming speed (C). Box plot (A) represents the 5th/95th percentiles (lower/upper whisker), the 25th/75th quartiles (box) and the median (horizontal line in box). Data (B and C) were graphed as means ± SEM (n=10;
*p<0.05, **p<0.01, vs. control group; one-way ANOVA with LSD post hoc test).

Figure 4. 1-bromopropane (1-BP) induced significant neuron loss in layer 5 in prelimbic cortex (PL).

(A) Representative photomicrographs (magnification, x10) of rat coronal sections showed NeuN-positive neuronal cells in a hemisphere of the brain. The boundaries of PL region were drawn with black lines and the strips of PL (200 µm in width) were outlined by blue lines. (B) Representative photomicrographs (magnification, x100) of the selected strips (200 µm in width) showed NeuN-positive neuronal cells in different layers of PL. (C) 1-BP induced significant and dose-dependent neuron loss in layer 5 of PL. However, numbers of neurons in layer 2/3 and layer 6 showed no significant difference among all groups. Data were graphed as means ± SEM (n=4; *p<0.05, **p<0.01, vs. control group; one-way ANOVA with LSD post hoc test).

Figure 5. (A) 1-bromopropane (1-BP) decreased neuroglobin protein level dose-dependently. Representative blots and quantification normalized to β-actin were shown. Data were expressed as means ± SEM (n=10; *p<0.05, **p<0.01, vs. control group; one-way ANOVA with LSD post hoc test). Ngb decrease in cerebral cortex was positively correlated with cognitive dysfunction induced by 1-BP. Ngb protein level in cerebral cortex showed significantly negative correlations with escape latency (B) and distance travelled (C), and positive correlations with percentage of time spent in target quadrant (D) and number of platform crossing (E). Data were graphed with
mean values and bidirectional error bars (SEM). Correlation analyses were performed with Spearman correlation coefficient.

Figure 6. 1-bromopropane (1-BP) suppressed glutamate cysteine ligase (GCL) activity and disrupted the redox homeostasis in cerebral cortex.

1-BP exposure resulted in decreased GSH content (A), increased GSSG content (B), decreased GSH/GSSG ratio (C) and suppression of GCL activity (D) in cerebral cortex. Absolute values were shown in left y axis (bars); whereas percentages of control value were shown in right y axis (lines). Data were expressed as means ± SEM (n=10; *p<0.05, **p<0.01, vs. control group; one-way ANOVA with LSD post hoc test).

Figure 7. Neuronglobin (Ngb) decrease in cerebral cortex was positively correlated with glutathione (GSH) depletion and GSH/oxidized glutathione (GSSG) ratio reduction.

Ngb protein level showed significantly positive correlations with GSH content (A) and GSH/GSSG ratio (B) in cerebral cortex. Data were graphed with mean values and bidirectional error bars (SEM). Correlation analyses were performed with Spearman correlation coefficient.

Figure 8. Cognitive dysfunction induced by 1-bromopropane (1-BP) was significantly positively correlated with glutathione (GSH) decrease and
GSH/oxidized glutathione (GSSG) ratio decrease in cerebral cortex.

GSH content of cerebral cortex showed significantly negative correlations with escape latency (A) and distance travelled (B), and positive correlations with percentage of time spent in target quadrant (C) and number of platform crossing (D). GSH/GSSG ratio of cerebral cortex showed significantly negative correlations with escape latency (E) and distance travelled (F), and positive correlations with percentage of time spent in target quadrant (G) and number of platform crossing (H). Correlation analyses (A, B, E, F) were performed with Spearman correlation coefficient, and correlation analyses (C, D, G, H) with Pearson correlation coefficient.

Figure 9. 1-bromopropane (1-BP) induced lipid peroxidation in cerebral cortex.

1-BP resulted in elevated levels of N-epsilon-hexanoyl-lysine (HEL) (A) and 4-hydroxy-2-nonenal (4-HNE) (B) modified proteins in cerebral cortex. Representative blots and quantification normalized to β-actin were shown. Data were expressed as means ± SEM (n=10; *p< 0.05, **p<0.01, vs. control group; one-way ANOVA with LSD post hoc test).

Highlights:

1-BP induced cognitive dysfunction and neuron loss in layer 5 of the prelimbic cortex.

1-BP decreased Ngb protein level in cerebral cortex dose-dependently.

1-BP led to GSH depletion and lipid peroxidation.

Cortical Ngb decrease might partly be attributed to oxidative stress.

Cortical Ngb decrease might have a role in 1-BP-induced neurotoxicity.