Research report

Carbon monoxide poisoning–induced delayed encephalopathy accompanies decreased microglial cell numbers: Distinctive pathophysiological features from hypoxemia–induced brain damage

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HIGHLIGHTS

- Demyelination from carbon monoxide persists longer than that caused by hypoxia.
- Microglia are inhibited by carbon monoxide but are activated by hypoxia.
- Carbon monoxide suppresses expression of microglial neurotrophic factors.

ABSTRACT

Carbon monoxide (CO) causes not only acute fatal poisoning but also may cause a delayed neurologic syndrome called delayed encephalopathy (DE), which occasionally occurs after an interval of several days to several weeks post-exposure. However, the mechanisms of DE have not been fully elucidated. This study aimed to clarify the pathophysiology of CO–induced DE and its distinctive features compared with hypoxic hypoxia. Rats were randomly assigned to three groups; the air group, the CO group (exposed to CO), and the low O\textsubscript{2} group (exposed to low concentration of O\textsubscript{2}). Impairment of memory function was observed only in the CO group. The hippocampus tissues were collected and analyzed for assessment of CO-induced changes and microglial reaction. Demyelination was observed only in the CO group and it was more severe and persisted longer than that observed in the low O\textsubscript{2} group. Moreover, in the CO group, decreased in microglial cell numbers were observed using flow cytometry, and microglia with detached branches were observed were observed using immunohistochemistry. Conversely, microglial cells with shortened branches and enlarged somata were observed in the low O\textsubscript{2} group. Furthermore, mRNAs encoding several neurotrophic factors expressed by microglia were decreased in the CO group but were increased in the low O\textsubscript{2} group. Thus, CO–induced DE displayed distinctive pathological features from those of simple hypoxic insults: prolonged demyelination accompanying a significant decrease in microglial cells. Decreased neurotrophic factor expression by microglial cells may be one of the causes of CO–induced DE.

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Abbreviations: CO, carbon monoxide; DE, delayed encephalopathy; dpe, days post-exposure; COHb, carboxyhemoglobin; HBO, hyperbaric oxygen; ROS, reactive oxygen species; O\textsubscript{2}Hb, oxyhemoglobin; PA test, passive-avoidance test; FCM, flow cytometry; MBP, myelin basic protein; MAG, myelin-associated glycoprotein; GST\textsubscript{\pi}, glutathione S-transferase pi 1; OPCs, oligodendrocyte progenitor cells; NG2, neuron-glial antigen 2; PDGFRA, platelet-derived growth factor receptor \alpha; NeuN, neuronal nuclei; SYP, synaptophysin; Syn1, synapsin1; Tau, Tau protein; PGP 9.5, protein gene product 9.5; Neuro D1, neurogenic differentiation 1; Tmem119, transmembrane protein 119; IGF, insulin-like growth factor; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; BDNF, brain-derived neurotrophic factor; PDGF-A, Platelet-derived growth factor-A; miRROS, mitochondria ROS; TNF-\alpha, tumor necrosis factor-\alpha; IL-1\beta, interleukin-1\beta; IL-6, interleukin-6; c-cas3, cleaved caspase 3; BID, BH3 interacting-domain death agonist; BAK, Bcl-2 homologous antagonist killer; BAX, Bcl-2-associated X protein; BAD, Bcl-2-associated death promoter; ppm, parts per million; ABP, arterial blood pressure; HR, heart rate; ABG, arterial blood gas

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1. Introduction

Carbon monoxide (CO) poisoning is generated accidentally by motor vehicles, appliances such as heaters, and household fires as its main sources, and causes the loss of consciousness or death in severe cases. The main pathophysiological mechanism of acute CO poisoning is hypoxia caused by compromised oxygen delivery resulting from the formation of Carboxyhemoglobin (COHb), which has 200 times greater affinity for hemoglobin than oxygen.

In addition to acute fatal poisoning, a delayed neurologic syndrome may occur after an interval of several days to several weeks called delayed encephalopathy (DE), also known as delayed neuropsychiatric sequelae. The symptoms of individual patients differ, e.g., memory loss, subtle cognitive deficits to severe dementia, psychosis, and parkinsonism. The rate of incidence is approximately 15 percent of severely poisoned patients (Ochi et al., 2014; Piantadosi, 2002; Roderique et al., 2015). Despite its use since the 1960s, the efficacy of hyperbaric oxygen (HBO) therapy, the therapy of choice for CO poisoning and prevention of DE (Ledingham, 1964; Sluijter, 1963), remains controversial (Roderique et al., 2015). However, available treatments with significant benefits besides HBO have not yet been established. One of the reasons given for this includes an incompletely elucidated mechanism for DE. The detailed mechanisms of DE cannot be explained only by tissue hypoxia resulting from COHb formation. Some other pathological entities such as reactive oxygen species (ROS) and nitric oxide are suggested to be potential mediators that worsen the insult (Piantadosi et al., 1997a; Thom et al., 1997; Thom et al., 2004).

In the brain, microglial cells constitutively search for abnormalities. Once they find injured myelin and nerves, microglia are activated and clear the debris in cooperation with macrophages derived from the bloodstream. The debris clearance by microglia is essential for remyelination and nerve regeneration (Neumann et al., 2009). Also, microglia are reported to support the survival and maturation of neuronal and glial cells by expressing neurotrophic factors (Domingues et al., 2016; Hoyos et al., 2014; Nakano et al., 2017; Nicholas et al., 2001; Nicholas et al., 2002; Ohya et al., 2007; Parkhurst et al., 2013; Pasquini et al., 2011; Reuss and von Bohlen und Halbach, 2003; Ueno et al., 2013; Zechel et al., 2010). Conversely, inflammation induced by activated microglia is detrimental to nerves and is known as secondary injury. Therefore, the function of activated microglia is often referred to as a “double-edged sword”. The contributions of microglial activation are reported as one of the mechanisms for the pathogenesis of DE (Thom et al., 2004). The cause of microglial activation in CO poisoning is thought to be the neuronal damage induced by tissue hypoxia. Indeed, hypoxic hypoxia also occasionally causes an encephalopathy known as delayed post-hypoxic leukoencephalopathy (Cree et al., 2018; Shprecher and Mehta, 2010). However, research comparing CO poisoning with hypoxic hypoxia and focusing on the relationship between CO-induced DE and immune reactions are limited.

Clarification of the mechanisms associated with CO-induced DE and its differences from the neuronal damage associated with hypoxic hypoxia may contribute to the development of novel therapeutics. In our study, we aimed to clarify the pathophysiological mechanisms of CO-induced DE and to compare the differences in inflammation and responses of microglial cells to hypoxic hypoxia.

2. Results

2.1. Significantly decreased passive-avoidance test latencies following CO exposure.

We observed the behavioral influences of CO or low oxygen exposure (Fig. 1). The PA test is a fear-aggravated test that is widely used to evaluate learning and memory function in CNS disorders. In this study, during the exposure, 70% of the CO group rats lost consciousness, whereas none of the air group rats or the low O2 group rats exhibited loss of consciousness. There were no changes in the test latencies of the air group or the low O2 group throughout the experiment duration. The latencies of only the CO group began to decrease after 3 dpe and were significantly decreased after 7 dpe compared with the baseline responses (day 0) or other treatment groups.

2.2. Long-term demyelination in the hippocampus in the CO group accompanied the loss of oligodendrocytes and oligodendrocyte progenitor cells.

It is well-known that CO induces demyelination (Law-Ye et al., 2017; Roderique et al., 2015; Shprecher and Mehta, 2010; Thom et al., 2004). Our qRT-PCR data showed that there was a significant decrease in mRNA expression encoding myelin basic protein (MBP) in the CO group and the low O2 group compared with the air group. Decreased MBP mRNA expression was observed in the CO group from 7 to 21 dpe, and was significant compared with the low O2 group at 21 dpe (Fig. 2Aa). Immunoblotting of hippocampal lysate samples corroborated the qRT-PCR data (Fig. 2Ba). The mRNA encoding myelin-associated glycoprotein (MAG), another protein of the myelin sheath, was also significantly decreased throughout the experimental duration in the CO group compared with other groups (Fig. 2Ab). The mRNA encoding glutathione S-transferase pi 1 (GSTπ), a marker of mature oligodendrocytes (Deshmukh et al., 2013), was also significantly decreased compared with the air group between 7 and 21 dpe, and compared with the low O2 group at 21 dpe (Fig. 2Ac). Significantly decreased GSTπ protein was observed with immunoblotting at 7–21 dpe compared with other groups (Fig. 2Bb). These data indicate demyelination after CO exposure, which is consistent with previous reports (Law-Ye et al., 2017; Roderique et al., 2015; Shprecher and Mehta, 2010; Thom et al., 2004). Moreover, these data suggest that the demyelination following CO exposure is more severe and lasts longer than the demyelination that occurs as a result of low oxygen exposure.

Oligodendrocytes develop from oligodendrocyte progenitor cells (OPCs) (Kang et al., 2010; Zhu et al., 2011). Therefore, we similarly examined the influences of CO exposure on OPCs. Neuro-glial antigen 2 (NG2) and platelet-derived growth factor receptor α (PDGFRα) are widely used as markers for OPCs. qRT-PCR data revealed that the mRNAs encoding both genes were decreased only in the CO group throughout the experimental duration, and was significant at 7 and 21 dpe compared with other groups (Fig. 2Ca andCb). Immunoblotting also revealed a significant decrease in NG2 protein at 7 dpe (Fig. 2D).

![Fig. 1. Analysis of learning and memory function after CO or low oxygen exposure. Changes in passive-avoidance test latencies for entrance to a dark compartment by each group of rats after Air/CO/low oxygen exposure. Open bars represent the mean ± SD, * indicate P < 0.05, **, and *** indicate P < 0.01, or 0.001, respectively.](image-url)
2.3. Axonal and neuronal damage after CO exposure

mRNAs of various neuronal and axonal markers were analyzed using qRT-PCR to investigate the effects of CO and low oxygen on neuronal cells. mRNA encoding some neuronal markers, including neuronal nuclei (NeuN) and synaptophysin (SYP), markers of neuronal cell bodies and presynaptic terminals, respectively, were significantly decreased in only the CO group at 7dpe (Fig. 3Aa and Ab). Synapsin 1 (Syn1) and TAU protein (TAU), markers of synaptic vesicles and axonal microtubules, respectively, were significantly decreased between 7 and 21dpe in the CO group (Fig. 3Ac and Ad). The mRNA of neuron cytoplasmic protein gene product 9.5 (PGP 9.5) was decreased throughout the experimental duration in the CO group (Fig. 3Ae).

Neuronal marker mRNA in the low O2 group showed no significant changes compared with the air group except for decreased PGP 9.5 at 3dpe. We also examined mRNA expression in neuronal progenitor cells to investigate their influences on immature neurons. The mRNA encoding neurogenic differentiation factor 1 (Neuro D1), a transcription factor that promotes neuronal development, was significantly decreased in the CO group throughout the experimental duration and also in the low O2 group at 3dpe (Fig. 3Af).

2.4. Hippocampal microglial cell numbers decrease following CO exposure.

Next, we examined microglial activation after CO exposure. In a previous report of CO–induced DE, microglial activation was observed at 3 weeks after CO exposure, suggesting that this activation may mediate DE (Thom et al., 2004). In our experiment, mRNAs encoding several microglia/macrophage markers, including Iba1 (Fig. 4Aa), transmembrane protein 119 (Tmem119; Fig. 4Ab), and CD11b (Fig. 4Ac) were analyzed using qRT-PCR. These markers were continuously decreased in the CO group until 21 dpe compared with the Air and Low O2 groups. The mRNA encoding Iba1 in the low O2 group exhibited a significant increase at 3 and 7 dpe (Fig. 4Aa). Immunoblotting indicated that CD11b protein was also significantly decreased at 7 and 21 dpe in the CO group (Fig. 4B). Based on these data, we examined the condition and number of microglial cells in the hippocampus at 7 dpe using flow cytometry (FCM). The gating strategy is illustrated in Fig. 4C. FCM revealed that the percentage of microglial cells among live cells was significantly decreased only in the CO group (Fig. 4D). These data demonstrated that microglial cells are significantly decreased in the CO group compared with the low O2 group.
2.5. Immunohistochemical analysis of microglial cells, OPCs, and neuronal cells in the hippocampus after CO or low oxygen exposure.

The dentate gyrus of the hippocampus was morphologically analyzed by immunohistochemical staining using antibodies to CD11b, NG2, and NeuN at 7dpe (Fig. 5). In the air group, abundant resting microglia were observed (Fig. 5Aa, yellow arrowhead). In the CO group, fewer microglia with detached branches were observed (Fig. 5Ba, yellow arrowhead). Activated microglia with shortened branches and enlarged somata were observed in the low O2 group (Fig. 5Ca, yellow arrowhead). Similar changes in microglial cells were observed in the frontal and parietal cortex (Supplementary Fig. 2).

Numerous NG2+ OPCs were observed in the air group (Fig. 5Ab). Some CD11b+/NG2+ cells were observed in the low O2 group but not in the air or CO groups (Fig. 5C, white arrowhead). NeuN+ neuronal cells in the dentate gyrus of the hippocampus were preserved in the air group but were scattered in the CO group (Fig. 5Ac and Bc, respectively, white arrow), in accordance with a previous report (Ochi et al., 2014). In the low O2 group, NeuN+ neuronal cells were decreased in number compared with the air group, but the decrease was milder than that in the CO group (Fig. 5Cc, white arrow).

2.6. The expression of neurotrophic factors after CO exposure in the hippocampus.

Microglial cells secrete neurotrophic factors and support the survival and maturation of neuronal and other glial cells. Based on the data indicating a decreased number of microglial cells, qRT-PCR was used to analyze the mRNA expression encoding some neurotrophic factors such as insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), hepatocyte growth factor (HGF), fibroblast growth factors 2 (FGF-2), brain-derived neurotrophic factor (BDNF), and platelet-derived growth factor- AA (PDGF-AA). IGF-1 mRNA was significantly decreased in the CO group between 3 and 21 dpe, and in low O2 groups at 7 dpe compared with the air group (Fig. 6Aa). IGF-2 mRNA in the CO group was significantly decreased compared with the air group between 3 and 21 dpe, and the low O2 group at 7 dpe, but gradually increased until 21 dpe. There was a significant decrease in the low O2 group at 3 dpe, followed by a gradual increase until 21 dpe (Fig. 6Ab). HGF mRNA in the CO group was significantly decreased compared with the air and low O2 groups between 3 and 21 dpe. The low O2 group showed a significant decrease at 3 dpe, followed by a gradual increase until 21 dpe (Fig. 6Ac). FGF-2 mRNA was significantly decreased in the CO group compared with the air group between 3 and 7 dpe and compared with the low O2 group at 7 and 21 dpe. Furthermore, the low O2 group was also significantly decreased compared with the air group at 3 and 7 dpe (Fig. 6Ad). BDNF mRNA in the CO group was significantly decreased at 3 and 7 dpe compared with the air group, and at 7 dpe compared with the low O2 group. A significant decrease in the low O2 group compared with the air group was observed at 3 dpe (Fig. 6Ae). PDGF-A mRNA was significantly decreased in the CO group, but not in the air and low O2 groups at 7 and 21 dpe (Fig. 6Af).

Next, to investigate whether the decreased mRNA expression of neurotrophic factors in hippocampus results from the decrease of microglial cells, we analyzed the mRNAs expression of these neurotrophic factors in microglial cells collected by magnetic cell sorting at 7dpe. All mRNAs of neurotrophic factors expressed by microglial cells in the CO group were significantly increased compared with the air and low O2 groups. mRNAs encoding IGF-1 and HGF expressed by microglial cells in the low O2 group were significantly increased compared with the air group. These data indicate that decreased expression of neurotrophic factor mRNA in the hippocampus after CO exposure may be attributable at least in part to the decreased expression by microglial cells.
2.7. Generation of reactive oxygen species and expression of pro-inflammatory cytokines after CO exposure in the hippocampus.

Microglia could exert detrimental effects on neurons and myelin sheaths through their production of ROS and pro-inflammatory cytokines. Therefore, we investigated whether mitochondrial ROS (mitoROS) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), and Interleukin-6 (IL-6) affect DE after CO exposure.

Generation of mitoROS by microglia in the hippocampus at 7 dpe was evaluated using flow cytometry. There were no significant differences in mitoROS generation by microglia between the three groups at 7 and 21 dpe and the decrease in the number of microglial cells was revealed by FCM at 7 dpe. (A) mRNA expression levels of microglial cell markers in the hippocampus at 3, 7, and 21 dpe. (B) CD11b immunoblots of rat hippocampus at 7 and 21 dpe. (C) Microglial cells in the hippocampus at day 7 were analyzed by flow cytometry. Live cells were gated based on forward scatter (FS) and side scatter (SS) values, followed by selection by the exclusion of Zombie NIR™ staining. (D) Microglial cells were identified using CD45-PE and CD11b/c-PE/cy7. The graphed qRT-PCR data are shown as the mean relative mRNA of n = 4/group normalized to GAPDH and compared with the naive hippocampal samples. β-actin was used as a loading control for immunoblotting. The immunoblotting graph represents the mean density of n = 4/group as a percentage of β-actin compared with the air group. The FCM images are representative of n = 5/group. Data are expressed as the mean ± SEM. *, **, and *** indicate P < 0.05, 0.01, or 0.001, respectively.

Fig. 4. The influence of CO or low oxygen exposure on microglial cells. Expression of mRNAs and proteins related to microglial cell markers were analyzed using qRT-PCR and immunoblotting. The mRNA and protein expression of microglial cell markers were decreased only in the CO group between 7 and 21 dpe and the decrease in the number of microglial cells was revealed by flow cytometric analysis at 7 dpe. (A) mRNA expression levels of microglial cell markers in the hippocampus at 3, 7, and 21 dpe. (B) CD11b immunoblots of rat hippocampus at 7 and 21 dpe. (C) Microglial cells in the hippocampus at day 7 were analyzed by flow cytometry. Live cells were gated based on forward scatter (FS) and side scatter (SS) values, followed by selection by the exclusion of Zombie NIR™ staining. (D) Microglial cells were identified using CD45-PE and CD11b/c-PE/cy7. The graphed qRT-PCR data are shown as the mean relative mRNA of n = 4/group normalized to GAPDH and compared with the naive hippocampal samples. β-actin was used as a loading control for immunoblotting. The immunoblotting graph represents the mean density of n = 4/group as a percentage of β-actin compared with the air group. The FCM images are representative of n = 5/group. Data are expressed as the mean ± SEM. *, **, and *** indicate P < 0.05, 0.01, or 0.001, respectively.

2.7. Generation of reactive oxygen species and expression of pro-inflammatory cytokines after CO exposure in the hippocampus.

Microglia could exert detrimental effects on neurons and myelin sheaths through their production of ROS and pro-inflammatory cytokines. Therefore, we investigated whether mitochondrial ROS (mitoROS) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), and Interleukin-6 (IL-6) affect DE after CO exposure.

Generation of mitoROS by microglia in the hippocampus at 7 dpe was evaluated using FCM. There were no significant differences in mitoROS generation by microglia between the three groups at 7 dpe (Supplementary Fig. 3Aa, Ab). The expression of mRNA and protein of pro-inflammatory cytokines in the hippocampus were also investigated by qRT-PCR (Supplementary Fig. 3B) and ELISA (Supplementary Fig. 3C), respectively. Furthermore, the three groups throughout the experimental duration showed no significant differences in hippocampal TNF-α mRNA in the hippocampus. However, IL-1β mRNA was decreased in the CO group at 7 dpe, and IL-6 mRNA was significantly decreased in CO group between 3 and 21 dpe and in the Low O₂ group at 3–7 dpe compared with air group. There were no significant differences between the three groups at the protein level regarding all investigated cytokines at 7 dpe. Also, we analyzed mRNAs of these pro-inflammatory cytokines expressed by the sorted microglial cells at 7 dpe. All mRNAs of pro-inflammatory cytokines expressed by microglial cells in the CO group were significantly decreased compared with the air and the low O₂ group. These data suggest that ROS and pro-inflammatory cytokines are not critical mediators of CO-induced DE.
2.8. CO exposed induces apoptosis in the hippocampus via the intrinsic apoptotic pathway. The influences of CO and low oxygen exposure on cell apoptosis were examined by immunoblotting using an antibody against cleaved caspase 3 (c-cas3). C-cas3 immunoblots exhibited a significant increase in the CO group at 7 dpe (Fig. 7A), suggesting widespread apoptotic cell death at 7 dpe in the hippocampus. Next, we examined mRNAs encoding apoptosis-related factors. A significant increase was observed in mRNAs encoding BH3 interacting domain death agonist (BID), Bcl-2 homologous antagonist killer (BAK), Bcl-2-associated X protein (BAX), and Bcl-2-associated death promoter (BAD) (Fig. 7Ba-Bd), which are the constituents of the intrinsic apoptotic pathway. Specifically, mRNAs encoding BID and BAK in the CO group were significantly increased between 7 and 21 dpe compared with the air and low O2 group. In the low O2 group, there was a significant increase in BID mRNA at 3 dpe, followed by a significant decrease between 7 and 21 dpe compared with the air group. No significant differences were observed in other mRNA expression levels in the low O2 group compared with the air group. These data suggest that mitochondria-mediated programmed cell death may be involved after CO exposure and lasts for at least 21 days.

Fig. 5. Immunohistochemical analysis of the hippocampus after CO or low oxygen exposure. Immunohistochemical analyses of the air group (A), the CO group (B), and the low O2 group (C) were performed at 7 dpe using antibodies against CD11b (red), NG2 (green), and NeuN (purple). Detached microglial branches were observed in the CO group (Ba, yellow arrowhead). Amoeboid-type microglia with enlarged somata were observed in the low O2 group (Ca, yellow arrowhead) and some of those microglia expressed NG2 on their membranes (C, white arrowhead). A decrease of NG2+ OPCs in the CO group was observed (Ab, Bb, Cb). Nuclear staining was performed using Hoechst 33,342 solution. Scale bars = 200 µm (Aa-d, Ba-d, Ca-d), 100 µm (Ae-g, Be-g, Ce-g).
3. Discussion

This research was focused on microglial function in CO–induced brain damage and compared CO–induced damage with hypoxemic hypoxia-induced brain damage side-by-side. In this study, we demonstrated for the first time that 1) demyelination induced by CO is more severe and persists longer compared with that induced by pure hypoxia; 2) microglial cells in the hippocampus were decreased after CO exposure; 3) neurotrophic factors are decreased in the hippocampus after CO exposure compared with low O2 exposure.

The impairment of memory and/or cognitive functions after CO poisoning is well-known (Mathieu et al., 1985; Min, 1986). In our experiment, we used the same conditions for CO exposure as previously reported (Ochi et al., 2014; Thom et al., 2004). The impairment of memory function was similarly observed after 7dpe in the CO group but not in the air or low O2 group. It is reported that DE caused by CO poisoning initiates development after one to two weeks of CO exposure (Ochi et al., 2014), which is consistent with our data. The impairment of memory function was observed only in the CO group but not in the low O2 group, indicating that the resulting pathophysiology differs between CO and low oxygen exposure. For this study, we set the O2 concentration of the low O2 group based on the O2Hb concentration from the analyzed arterial blood samples of the CO group. In the case of CO poisoning, despite decreased O2 supply due to the inhibition by COHb, SpO2 or PaO2 exhibit no changes during CO inhalation as indicated in the vital graph and ABG data (Supplementary Fig. 1 and Supplementary Table 1). Therefore, SpO2 or PaO2 are inadequate for monitoring hypoxia during CO exposure. For this reason, we used O2Hb as an index to assess the degree of hypoxia in the case of CO inhalation.

Glial cells other than astrocytes are thought to be vulnerable to hypoxia (Lyons and Kettenmann, 1998). In our experiment, a decrease in the mRNAs and proteins for the marker of a mature myelin sheath was observed in the CO group. It has been reported that CO induces demyelination (Law-Ye et al., 2017; Roderique et al., 2015; Shprecher and Mehta, 2010; Thom et al., 2004), and our data are consistent with these reports. Demyelination in the hippocampus leads to cognitive impairment and affects memory function (Falck et al., 2016; Xu et al., 2017). As reported previously, the cognitive impairment observed in the CO group may be caused, in part, by demyelination.

Following injury or myelin damage, NG2 and PDGFRα-expressing adult OPCs differentiate into myelinating oligodendrocytes, but not astrocytes or neurons (Kang et al., 2010; Zawadzka et al., 2010). In our experiment, mRNAs and protein for the marker of OPCs were also significantly decreased only in the CO group but not in the low O2 group, and this reduction lasted for at least 21 days. This decrease in OPCs may be responsible for the observed continued demyelination, and consequently, the retardation of remyelination in the CO group. The decrease of OPCs may be more critical for pathogenesis of DE rather than the decrease of mature myelin in CO poisoning, which may be associated with the disappearance of memory dysfunction in the low O2 group. It has been reported that the memory dysfunction caused by pure hypoxia is not progressive (Kesner and Hopkins, 2001), which corroborates our hypothesis.

The neuronal damage in the hippocampus leads to memory and cognitive impairments (De Jong et al., 1999; Pappas et al., 1996). The experiment using a cell line demonstrated CO–induced apoptosis at a concentration of 1000 parts per million (ppm) (Tofighi et al., 2006). In other reports, a significant decrease in neuronal cells in the cortex and
hippocampus after CO exposure was reported (Ochi et al., 2014; Piantadosi et al., 1997b). In the present research, qRT-PCR revealed a significant decrease in the expression of mRNAs encoding some neuronal markers in the hippocampus at 7–21 dpe in the CO group compared with other groups. Damage to NeuN⁺ cells in the CO group was observed in immunohistochemical studies. These findings indicate that the neuronal damage induced by CO exposure is more severe than that induced by pure hypoxia.

Microglial activation revealed by immunohistochemical study and qRT-PCR has been reported and is posited to contribute to DE after CO exposure (Dong et al., 2015; Thom et al., 2004). Activated microglia called amoeboid-type microglia have relatively shortened dendrites and enlarged somata as compared with resting microglia called ramified-type or surveying microglia (Kettenmann et al., 2013). We predicted that microglial activation would induce critical neuronal and myelin damage in the hippocampus in our CO exposure model, as reported elsewhere (Thom et al., 2004). However, FCM revealed a significant decrease in the number of microglial cells only in the CO group. Immunoblotting and qRT-PCR also corroborated the evidence provided by FCM. Immunohistochemical studies showed microglial cells with detached dendrites in the CO group, but amoeboid-type microglia in the low O₂ group. These findings were also observed in cerebral cortices, suggesting that the damage and decrease of microglial cells may occur widely in the brain.

Moreover, CD11b⁺/NG2⁺ microglial cells were observed only in the low O₂ group. Activated microglia reportedly express NG2 on their membranes in the pathological brain, such as in cerebral infarction and Parkinson's disease, and NG2⁺ microglia are thought to be engaged in phagocytosis (Aono et al., 2017; Sugimoto et al., 2014). These data indicate pathophysiological differences in microglial reactions to CO and low oxygen exposure, as evidenced by the decrease in microglial cell number in the CO group, and microglial activation in the low O₂ group.

In the CNS, various neurotrophic factors that support the development, survival, and repair of the nervous system, such as IGFs, HGF, FGF, BDNF, and PDGF-AA are released by microglial cells. IGF-1 secreted by microglia is reported to be important for the survival of OPCs and neuronal cells (Pang et al., 2007; Ueno et al., 2013). IGF-2 is a trophic factor expressed by microglia to support the survival and maturation of OPCs (Domingues et al., 2016; Hoyos et al., 2014; Nicholas et al., 2001; Nicholas et al., 2002; Pasquin et al., 2011). HGF is known to suppress neuroinflammation, protect neuronal cells in the hippocampus, and increase OPCs (Nakano et al., 2017; Ohya et al., 2007). FGFs, especially FGF-2, is essential for neuronal differentiation and survival in the developing and mature brain and is thought to be involved in the regulation of memory and learning processes (Reuss and von Bohlen und Halbach, 2003; Zechel et al., 2010). Microglia-derived BDNF is essential for synaptic plasticity and has a critical role in learning and memory functions (Parkhurst et al., 2013). In our experiment, the expression of mRNAs encoding those neurotrophic factors was significantly decreased only in the CO group at some time points during the experimental duration. At the same time, activated microglial cells can injure neuronal cells and myelin through their release of ROS and pro-inflammatory cytokines. However, expression of these detrimental factors was not observed in the hippocampus after CO exposure. Furthermore, expression of mRNA for these cytokines by isolated microglial cells were significantly suppressed in the CO group at 7 dpe. Accordingly, ROS or pro-inflammatory cytokines may not have

![Fig. 7. The apoptosis pathway after CO or low oxygen exposure. Apoptosis in the hippocampus after CO or low oxygen exposure was analyzed by immunoblotting and the apoptotic pathway was investigated using qRT-PCR. Apoptosis was recognized in the hippocampus in the CO group at 7 dpe. (A) Immunoblot of cleaved caspase-3 in the hippocampus at 7 dpe. β-actin was used as a loading control. The graph represents the mean density of n = 4/group as a percentage of β-actin compared with the air group. (B) The intrinsic apoptotic pathway was suspected as one of the pathways responsible for CO-induced apoptosis. The graphed data are shown as the mean Relative mRNA of n = 4/group normalized to GAPDH and compared with the naïve hippocampal samples. Data are expressed as the mean ± SEM. *, **, and *** indicate P < 0.05, 0.01, or 0.001, respectively.](image-url)
played critical roles in the neuronal cell loss and demyelination in the CO-induced DE. The damage and decreased number of microglia presumably lead to the decrease of neurotrophic factors, while hindering remyelination and the survival of neuronal cells and OPCs. Although further investigation is required, supplementation with neurotrophic factors may be a promising therapeutic strategy.

It is reported that some neurotrophic factors protect neuronal cells, OPCs, and other cells from apoptosis by reducing the expression of Bax or enhancing the expression of B-cell lymphoma (Bcl)–2 protein (Nakagami et al., 2002; Pang et al., 2007; Schabitz et al., 2000). It has also been reported that CO induces apoptosis via mitochondrial dysfunction (Brown and Plantadosi, 1990) where the Bcl–2 family of genes is involved (Green and Kroemer, 2004). Our data indicate that CO exposure induces apoptosis in the hippocampus which is more severe than the apoptosis associated with low oxygen exposure, even at 7 dpe. Additionally, qRT-PCR data revealed that CO induces activation ofBid, Bak, and Bax, the constituents of the intrinsic apoptotic pathway, which eventually leads to the activation of caspase 3 (Bajwa et al., 2012). The increase in these factors is continuous for a long duration after even a single exposure to CO; however, low oxygen exposure does not affect this apoptotic pathway. These data suggest that mitochondria-mediated cell death via the intrinsic apoptotic pathway is responsible, in part, for the pathogenesis of DE following CO poisoning, and may result in part from decreased neurotrophic factors following apoptosis of microglial cells.

4. Conclusions

This study demonstrated that CO intoxication caused not only degeneration of neuronal cells but also that of glial cells. Decreased myelin proteins and NG2 indicated marked degeneration of oligodendrocytes and OPCs. Furthermore, this study demonstrated a marked decrease of microglial cell numbers in CO-exposed brain tissues. This microglial degeneration accompanied a decreased expression of neurotrophic factors and proinflammatory cytokines. These degenerative changes in glial cells were quite specifically observed only for CO-intoxication, but not in hypoxic brains. Among the changes in the CO-exposed brain, we suspected causal involvement of the decreased neurotrophic actions by microglia in the degeneration of neurons, OPCs, and oligodendrocytes. Therefore, protection of microglia from CO–induced damage or supplementation with neurotrophic factors may become a promising strategy for the therapy or prevention of CO–induced DE.

5. Materials and methods

All experimental procedures were performed according to the Guidelines for Animal Experimentation of Ehime University Graduate School of Medicine that were established in accordance with the guidelines published in the National Institute of Health Guide for the Care and Use of Laboratory Animals. Rats were housed in plastic cages at ambient temperature with a 12-h light-dark cycle and free access to food and water.

5.1. Animals and surgical procedures

5.1.1. Vital data and arterial blood gas data sampling

Adult male Wistar rats (8–10 weeks old, body weight 260–300 g; Charles River Laboratories, Yokohama, Japan) were used for all experiments. Rats were randomly assigned to three groups: the control group (air group); the CO-exposure group (CO group); and the low oxygen exposure group (low O2 group). Rats were anesthetized with 3% sevoflurane and intubated with a 16-gauge cannula (B. Braun Aesculap Japan, Tokyo, Japan) for mechanical ventilation. The femoral artery was exposed, and a 24-gauge cannula was inserted for arterial blood pressure (ABP) monitoring, heart rate (HR) monitoring, and arterial blood gas (ABG) analysis. Two gas cylinders containing 1000 ppm or 3000 ppm of CO were prepared beforehand. The air group rats were mechanically ventilated with air for 60 min. The CO group rats were mechanically ventilated with air mixed with CO at a concentration of 1000 ppm for 40 min, followed by 3000 ppm for 20 min. The low O2 group rats were mechanically ventilated with air mixed with N2 to reduce the O2 concentration to achieve an equivalent degree of hypoxia induced by CO inhalation. Oxyhemoglobin (O2Hb) was used as an index of hypoxia level because SpO2 or PaO2 are inadequate for monitoring the severity of CO exposure. SpO2, inspired gas concentration, and ABG were analyzed with Mouse Ox plus™ (Starr Life Sciences Corp., PA, USA), HT-1300 N™ (HODAKA CO., LTD., Osaka, Japan), andABL725™ (RADIOMETER MEDICAL ApS, Tokyo, Japan), respectively. ABP, HR, and SpO2 were recorded every minute The data are shown as supplementary data (See Supplementary Fig. 1). Exposed CO or O2 concentration measurements and ABG analysis were performed at 0, 40, and 60 mins after the start of exposure (See Supplementary Table 1). The O2Hb concentrations of the rats treated with 1000 ppm or 3000 ppm CO were matched with those treated with 8% or 4% O2, respectively.

5.1.2. CO and hypoxia exposure

Based on collected arterial blood gas analysis data, CO, and low oxygen exposure were performed as described elsewhere (Ochi et al., 2014; Thom et al., 2004). In brief, rats assigned to the CO group were exposed to 1000 ppm CO for 40 min, followed by 3000 ppm CO until loss of consciousness occurred, or for up to 20 min in a 7.6-L chamber (250 × 190 × 160 mm, KN-1010-L, Natsume Seisakusho, Tokyo, Japan). Low oxygen exposure was performed in the same chamber with an O2 concentration of 8% for 40 min, followed by exposure to 4% O2 for up to 20 min. Control rats were exposed to room air for 60 min. The concentration of CO and O2 were monitored for the duration of the experiments.

Rats were then moved to an air-conditioned room to regain consciousness. Rats were perfused transcardially with 4% paraformaldehyde in PBS containing 2 mM MgCl2 under deep anesthesia for immunohistochemical analysis or sacrificed for tissue collection at 3, 7, or 21 days post-exposure (dpe). Whole brains were excised, and hippocampal tissues were collected for further analysis.

5.2. The passive-avoidance test

Learning and memory function in each group of rats were evaluated using the step-through passive-avoidance test (PA test) as previously described (Ochi et al., 2014). The apparatus consisted of an illuminated (450 × 270 × 260 mm) and a dark (450 × 270 × 260 mm) compartment on a grid floor, separated by a sliding door (MPB-M001; Melquest, Toyama, Japan). In the training session before CO/hypoxia exposure, each rat was placed in the illuminated compartment and allowed to explore for 20 s. The sliding door was then opened, and the step-through latency for rats to enter the dark compartment with all four paws was measured. The door was immediately closed upon entry into the dark compartment. At least two additional trials were conducted until rats entered the dark compartment within 20 s. After the door was closed in the last trial, a foot-shock (1 mA for 5 s) was delivered through the grid floor with a constant current shock generator (SG-100; Melquest). The rats that entered the dark compartment within 300 s in the training session in spite of the first foot-shock were given a subsequent foot-shock, which was repeated until they failed to enter the dark compartment. At 24 h after the training session, it was confirmed that the rats would not enter the dark compartment within 300 s, and then they were exposed to air, CO, or low oxygen (Day 0). The step-through latency was recorded as “300 s” when rats did not enter the dark compartment within 300 s. The PA tests were performed at 0, 3, 7, 14 and 21 dpe.
5.3. RNA isolation and real-time RT-PCR analysis

The collected hippocampal tissues were homogenized in QIAzol lysis reagent (Qiagen, Hilden, Germany) and total RNA was extracted using an RNeasy lipid tissue Mini Kit (Qiagen). cDNA was synthesized from 1 µg DNase I-treated RNA by reverse-transcription using a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantitative real-time RT-PCR (qRT-PCR) analysis was performed in triplicate using an MJ mini instrument (Bio-Rad, Hercules, CA, USA) with SYBR™ Green Real-time PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan). PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For naïve hippocampal samples unexposed to CO or low O₂, a threshold cycle (Ct) value of 51 was assigned to allow calculations. All gene-specific mRNA expression values were normalized to GAPDH levels. Relative mRNA levels were quantified using the Delta-Delta Ct method. The primer sequences for each gene are listed in Supplementary Table 2.

5.4. Immunohistochemistry

After immersing the fixed brains in 20% sucrose in PBS overnight, the brains were rapidly frozen in powdered dry ice. Brains were serially sliced into 10-µm-thick coronal sections using a cryostat. The sections were rinsed with Tris-buffered saline (TBS) and then permeabilized and blocked with TBS containing 0.1% Tween 20 and 1 mg/ml BSA. The sections were then incubated with primary antibodies listed in Supplementary Table 3 overnight. The immunoreaction was visualized using Alexa Fluor™ 488- (Thermo Fisher Scientific Inc.), Alexa Fluor™ 549- (Thermo Fisher Scientific Inc.), and Cy5- (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) labeled secondary antibodies. Hoechst 33258 solution (Sigma-Aldrich, St. Louis, MO, USA) was used for nuclear counter-staining. The sections were observed using a BZ-9000 scanning fluorescence microscope (Keyence, Osaka, Japan).

5.5. Immunoblotting

The hippocampal tissues were homogenized and lysed in Laemmli’s sample solution. The lysates were electrophoresed with 5–20% of Tris-glycine gel and transferred onto nitrocellulose membranes using Horize BLOT 2M-R™ (ATTO Co., Tokyo, Japan). The membrane was blocked in 5% nonfat milk (Bio-Rad) and incubated for 16–18 h at 4 °C with the primary antibodies listed in Supplementary Table 3, followed by incubation for 4 h at ambient temperature with the species-specific horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Boston, MA, USA). The blots were developed with an enhanced chemiluminescence system (GE Healthcare UK Ltd., Buckinghamshire, England) and LuminoGraph I™ (ATTO Co.). β-actin was used as a loading control. The bands were digitized and quantitated using Image J 1.43u software (National Institutes of Health, Bethesda, MD, USA).

5.6. Quantification of pro-inflammatory cytokines in hippocampal tissue

Pro-inflammatory cytokine proteins in the hippocampus were quantified using an ELISA kit (R&D Systems, Minneapolis, MN, USA). Before the assay, hippocampal tissues were homogenized in N-PER™ Neuronal Protein Extraction Reagent (Thermo Fisher Scientific Inc.) at a concentration of 100 mg/200 µl and incubated 20 min at 4 °C. After centrifugation, supernatants were prepared and the ELISA assay was performed in duplicate.

5.7. Tissue dissociation for single-cell suspensions

At 7 dpe, rats were perfused transcardially with cold PBS under deep anesthesia to remove their blood, and whole brains were excised at 7 dpe. Hippocampal tissues were collected for the production of dissociated single cells for flow cytometry (FCM) according to instructions included in an Adult Brain Dissociation Kit (ABDK) (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, collected hippocampal tissues were transferred to tubes containing 2 ml of an enzyme mixture and dissociated using a program “37 °C,ABDK 01” for a gentle MACS dissociator with heaters™ (Miltenyi Biotec). The suspensions were passed through a MACS Smart Strainer™ (100 µm pore size) (Miltenyi Biotec) to remove large aggregates. Debris and contaminated erythrocytes were removed using debris removal and red blood cell removal solutions in ABDK.

5.8. Flow cytometry

The prepared single-cell suspensions were diluted to about 1×10⁶ cells/100 µl with PBS containing 2 mM EDTA and 2% fetal bovine serum (FBS), followed by incubation with anti-CD32 antibody Fc blocker (BD Pharmingen, Franklin Lakes, NJ, USA) for 20 min on ice to block Fc receptors. The suspensions were incubated with fluorescence-labeled antibodies listed in Supplementary Table 3 for 30 min on ice. To detect generated mitochondrial reactive oxygen species (mitoROS), cells were incubated with MitoROS 520 (AAT Bioquest, Sunnyvale, CA, USA) for 20 min at 37 °C. After rinsing, cells were resuspended in PBS and subjected to flow cytometry using a Gallios™ flow cytometer (Beckman Coulter, Tokyo, Japan). Propidium Iodide (BioLegend, San Diego, CA, USA) or Zombie NIR™ (BioLegend) were used for cell viability. FlowJo Software™ version.7.6.5 (Tree Star, Inc., Ashland, OR, USA) was used for data analysis.

5.9. Magnetic cell sorting (MACS) of microglial cells from adult hippocampus

The procedure was performed according to the instructions included with the CD11b/c-microbeads antibody (Miltenyi Biotec). In brief, the prepared single cell suspensions were diluted to about 1×10⁶ cells/80 µl with PBS containing 2 mM EDTA and 2% FBS, followed by incubation with 20 µl of anti-CD11b/c-microbeads antibody for magnetic labeling for 10 min, and with anti-CD11b/c-PE/cy7 and anti-CD45-PE for 5 min on ice. After rinsing, magnetically labeled microglial cells were separated with OctoMACS™ Separator (Miltenyi Biotec) and MS columns (Miltenyi Biotec). The columns were removed from the separator and magnetically labeled microglial cells were collected by flushing out with PBS containing 2 mM EDTA and 2% FBS. An aliquot of the suspensions was used for the purity check. Total RNA from collected cells was extracted using RNeasy Micro Kit (Qiagen) and cDNA was synthesized using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (TOYOBO).

5.10. Statistical analysis

Data were analyzed using GraphPad Prism™ 5.02 statistical software (GraphPad Software, Tokyo, Japan). The results were expressed as the mean ± SEM. A two-tailed Student’s t-test was used for comparisons of two groups. Analysis of variance with Tukey’s post hoc test was used for comparisons of three or more groups. Significance was determined by p values < 0.05.

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Conflict of interest

None.
