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Involvement of extracellular ascorbate and iron in hydroxyl radical generation in rat striatum in carbon monoxide poisoning

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ABSTRACT

Carbon monoxide (CO) poisoning stimulated generation in rat striatum of toxic hydroxyl radicals (•OH), which might participate in the CO-induced neuronal injury. Since an increase in extracellular ascorbate (AA) stimulated •OH generation in the presence of endogenous metals, including iron, in rat striatum in vivo, we examined the role of extracellular AA in •OH generation due to CO poisoning in the present study. The CO-induced •OH generation in the striatum was strongly suppressed by intrastriatal administration of active, but not inactivated, AA oxidase, which degrades extracellular AA. In addition, CO poisoning caused a significant increase in extracellular AA in rat striatum, suggesting a role of extracellular AA in the CO-induced •OH generation. However, the time-course of changes in extracellular AA could not be completely superimposed on that of the CO-induced •OH generation. On the other hand, the CO-induced •OH generation was completely suppressed by an iron chelator, deferoxamine. These findings suggest that •OH generation in rat striatum due to CO poisoning may involve both extracellular AA and chelatable iron.

1. Introduction

There are a number of reports concerning accidental and intentional carbon monoxide (CO) poisoning in humans in various countries (Henry et al., 2006; Raub et al., 2000; Theilade, 1990). Autopsy has revealed that CO poisoning injures various brain regions, including the cerebral cortex, globus pallidus, caudate putamen and hippocampus (Ginsberg, 1980; Lapresle and Fardeau, 1967). Further, neuropsychiatric abnormalities, including parkinsonism and dementia, with abnormal images of those brain regions in computed tomography or magnetic resonance imaging (MRI), have been reported in survivors of acute CO poisoning (Chang et al., 1992; Choi, 1983; Choi and Cheon, 1999; O'Donnell et al., 2000). We found that hydroxyl radical (•OH) generation was stimulated in the striatum of CO-poisoned rats (Hara et al., 2004). The stimulation of •OH generation is likely to participate in the cascade leading to CO-induced neuronal injury, since •OH is the most toxic among reactive oxygen species (ROS) and oxidative stress is associated with neuronal injury due to brain insults, including brain ischemia and trauma (Gilgun-Sherki et al., 2002; Leker and Shohami, 2002; Lewén et al., 2000). CO poisoning causes increases in extracellular glutamate (Glu) (Hara et al., 2004) and dopamine (DA) (Hara et al., 2002), both of which could lead to ROS generation in the striatum (Laplanche et al., 2000; Stokes et al., 1999). It also suppresses striatal production of nitric oxide (NO) in association with a decrease in extracellular L-arginine, the substrate of NO synthase (NOS) (Hara et al., 2003), under conditions where NOS may generate ROS instead of NO (Stuehr et al., 2001). However, blockade of Glu receptors or DA synthesis had little or no effect on CO-induced •OH generation (Hara et al., 2004). In addition, two different NOS inhibitors, N^G-nitro-L-arginine methyl ester and N^G-monomethyl-L-arginine, suppressed and enhanced the CO-induced •OH generation, respectively, and a calmodulin inhibitor, W7, which decreases NOS activity (Ohashi et al., 2007), had little effect on •OH generation (Hara et al., 2007). It is unlikely that the Glu, DA and NO systems are directly associated with the stimulation of •OH generation by CO poisoning.

Chen et al. (2007, 2008) found that, in normal rats, parenteral, but not oral, administration of ascorbic acid (AA; an antioxidant well-known as vitamin C) overwhelmed the homeostatic control of AA, leading to a striking AA increase in blood and in the extracellular fluid of peripheral tissues. Further, ROS generation was stimulated dependent on the increased AA in the latter location, but not the former, suggesting the prooxidant action of AA in vivo under normal physiological circumstances. We demonstrated that such a prooxi-

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dant action of AA was elicited in vivo by the increase in extracellular AA in rat striatum following intraperitoneal administration of dehydroascorbate (DHA) (Hara et al., 2009), although abundant AA exists in the brain and its major role is considered to be as a contributor to the defense system against oxidative stress, i.e., an antioxidant (Rice, 2000). On the other hand, it has been proposed that intracellular AA is released to the extracellular space in association with uptake of extracellular Glu via the transporter system in the brain (Rice, 2000). Based on the increase in extracellular Glu due to CO poisoning (Hara et al., 2004), it seems likely that extracellular AA in the striatum is increased in CO poisoning, resulting in stimulation of •OH generation. In the present study, we examined this hypothesis by means of brain microdialysis.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 230–270 g, were purchased from Charles River (Kanagawa, Japan). Animals were acclimated with free access to food and water in a facility with controlled temperature (22–24 °C) and humidity (50–60%), on a 12-h/12-h light/dark cycle (lights on between 8:00 and 20:00 h), for 1 week before all of the experiments.

The experimental protocol of this work was approved by the Tokyo Medical University Animal Care Committee and all experiments were performed in accordance with the Japanese Animal Research Association standards as defined in the Guideline for Animal Experiments and the Guiding Principle in the Use of Animals in Toxicology.

2.2. Chemicals

Pure (>99.9%) CO was purchased from Sumitomo Seika Chemicals (Tokyo, Japan). AA oxidase (AAO; 200 units (U), 245 U/mg protein) was from Wako Pure Chemicals (Tokyo, Japan). Deferoxamine (DFO) and ammonium iron (II) sulfate were from Sigma–Aldrich (St. Louis, MO, USA). Bathophenanthroline sulfonate (BPS) was from Dojindo (Kumamoto, Japan). Physiological saline was from Otsuka Pharmaceuticals (Tokyo, Japan). All other chemicals of analytical grade were from Wako Pure Chemicals or Nacalai Tesque (Kyoto, Japan).

2.3. Stereotaxic surgery and brain microdialysis

Stereotaxic surgery and brain microdialysis were performed according to the methods previously reported (Hara et al., 2009). A microdialysis probe with a 3-mm cellulose membrane (A-I-8-03; Eicom, Kyoto, Japan) was inserted into the striatum (coordinates of the tip of the probe; 0.2 mm AP, 3.0 mm L, 6.5 mm DV) (Paxinos and Watson, 1998) through the guide cannula, which had been implanted under pentobarbital anesthesia (50 mg/kg, i.p.) at least 5 days before. The dialysis probe was perfused with a modified Ringer solution (147 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$) in the presence (for •OH) or absence (for AA) of 5 mM sodium salicylate at a flow rate of 2 μL/min. When AAO was directly administered to the striatum, we used the MI-A-I-8-03 probe equipped with a thin fused-silica needle, and the tip of the needle was located adjacent to and in the middle of the membrane. The locations of the dialysis probes were verified after each experiment.

2.4. CO exposure and drug administration

CO exposure was performed according to the method previously reported (Hara et al., 2002). The plastic chamber (26.5 cm in diameter, 28.5 cm in height) containing the rat was enclosed with a cover, except for a hole through which the perfusion tubes passed. Room air containing pure CO was introduced into the chamber at a flow rate of 8 L/min. The concentration of CO in the chamber was adjusted to 3000 ppm by using a gas flow regulator (Koflok, Osaka, Japan), based on the readings from a CO monitor (CM-525HB; Gastec, Kanagawa, Japan). Such CO exposure for 40 min resulted in CO poisoning with over 70% blood carboxylhemoglobin level (Hara et al., 2002).

The commercial AAO was prepared for direct administration into the striatum as previously reported (Hara et al., 2009). The AAO was dissolved in 500 μL of ice-cold saline, and the solution was dialysed with a cellulose ester membrane (Spectra/Por CE Float A Lyze; molecular cut-off, 10,000 Da; Spectrum Laboratories, Rancho Dominguez, CA, USA) against 2 L of 4 mM phosphate buffer (pH 7.4) at 3 °C for 5 h, and thereafter, against 2 L of saline at 3 °C for 5 h. The resultant AAO solution was stored at −80 °C and diluted with saline immediately before administration. Direct administration of AAO to the striatum was done through the needle of the MI-A-I-8-03 probe, by using an ESP-32 microsyringe pump (Eicom). The AAO solution (18.2 mU/μL, 111 U/mg protein) was administered at 0.02 μL/min for 40 min (14.6 mU/0.8 μL/rat). A possible effect of AAO independent of its enzymatic activity was examined by the administration of AAO inactivated by storage at 50 °C for 48 h.

DFO was dissolved in the perfusing solution at 1 mM and administered to the striatum through the membrane of the probe throughout the experimental period.

2.5. Determination of •OH generation

•OH generation was estimated by measuring the extracellular levels of 2,3-dihydroxybenzoic acid (2,3-DHBA) formed through non-enzymatic hydroxylation of salicylic acid by •OH (Ingelman-Sundberg et al., 1991), according to the protocol of Teismann and Ferger (2000), with modifications (Hara et al., 2004). The dialysate (40 μL) was collected into an autoinjector (EAS-20; Eicom) every 20 min, and injected into an inert HPLC system (Eicom) equipped with an electrochemical detector (ECD-300; Eicom) consisting of a graphite working electrode at +500 mV vs. an Ag/AgCl reference electrode. Separation was done on an Eicompac SC-5ODS column (2.1 mm × 150 mm) at 25 °C with a mobile phase consisting of 100 mM sodium phosphate buffer (pH 6.0) containing EDTA·2Na (5 mg/L) and 2% methanol, at a flow rate of 230 μL/min.

2.6. Determination of extracellular AA

Extracellular AA was determined, as previously reported (Hara et al., 2009). The dialysate (40 μL) for every 20 min period was collected into a solution (560 μL) containing 0.1 M phosphate buffer (pH 3.5) and 1 mM EDTA in a dark plastic tube on ice. The AA concentration in the diluted dialysate was determined by using the above HPLC-ECD system at +450 mV. Separation was done on an Eicompac AC-GEL column (2.0 mm × 150 mm) at 25 °C with a mobile phase consisting of 100 mM sodium phosphate buffer (pH 6.0), EDTA·2Na (5 mg/L), hexadecyltrimethylammonium bromide (300 mg/L) and 30% methanol, at a flow rate of 120 μL/min.

2.7. Determination of extracellular chelatable iron

The extracellular level of chelatable iron was measured by the method of Nilsson et al. (2002) with modifications, as follows. The dialysate (40 μL) collected into a plastic tube for every 20 min period was mixed with 1 μL of 41 mM BPS, an iron chelator. After 10 min, the mixture was manually infused into the loop (20 μL) of an Eicom AS-10 autoinjector by using a disposable pipette tip connected with a 1-mL plastic syringe to fill the loop with the mixture, and then injected into a constant flow (140 μL/min) of Milli Q water by using an Eicom ENO-10 pump system. The absorbance at 535 nm was measured with a Hitachi UV–VIS L-7420 detector. There was no metal contact throughout the flow pathway. The peak area was calculated with PowerChrom software (Eicom). As shown in Fig. 1, the calibration curve using ammonium iron (II) sulfate dissolved in the perfusing solution as the standard was linear between 0 and 40 pmol/40 μL (1 μM) with the detection limit of 2 pmol/40 μL (0.05 μM), suggesting that our method is more sensitive than that of Nilsson et al. (2002).

2.8. Statistics

Changes in extracellular AA and 2,3-DHBA were expressed as a percentage of the respective basal levels, which were determined by averaging three consecutive dialysate samples in individual animals before various treatments. Data were expressed as means ± SEM obtained from five or six rats, and analyzed by using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple-group comparisons or Student's *t*-test for two-group comparisons. The interaction between two different treatments was analyzed by two-way ANOVA.

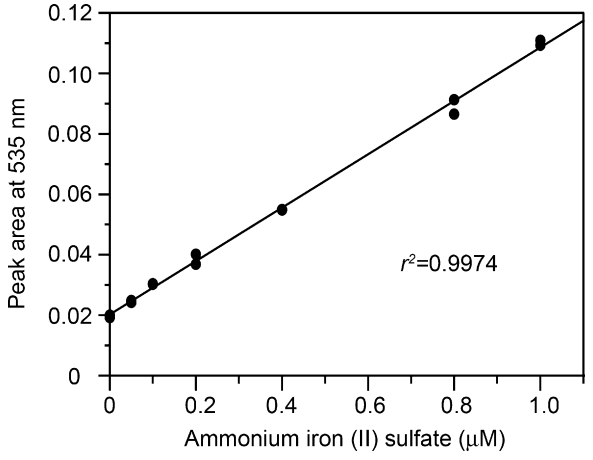

Fig. 1. Calibration curve of chelatable iron with BPS by using ammonium iron (II) sulfate as the standard.

3. Results

3.1. Effect of intrastriatal administration of AAO on the CO-induced increase in extracellular 2,3-DHBA

We have shown that administration of active and inactivated AAO into the striatum decreases the basal level of extracellular 2,3-DHBA, but the effect of active AAO is stronger than that of inactivated AAO (Hara et al., 2009). Here, we examined the effect of active and inactivated AAO on the CO-induced •OH generation in rat striatum. The effects of active and inactivated AAO were superimposed on those in the absence of CO exposure, as shown in Fig. 2, and statistically analyzed. In the rats with administration of inactivated AAO into the striatum during CO exposure, extracellular 2,3-DHBA in the striatum was remarkably increased during the last 20 min of CO exposure and the following period (immediately after termination of CO exposure) (Fig. 2). The extracellular 2,3-DHBA during these periods and also during the 20-min period following the latter period was significantly higher than that in the case of inactivated AAO alone without CO exposure (Fig. 2). In contrast, in the rats exposed to CO in combination with active AAO, extracellular 2,3-DHBA was decreased, but not increased, during those periods, and thereafter, further decreased to a level much lower than the basal level, tracing the levels in animals treated with active AAO alone (Fig. 2). Extracellular 2,3-DHBA in the rats treated with CO plus active AAO was significantly lower than that in rats exposed to CO in combination with inactivated AAO from the last 20 min of CO exposure through to the end of the experimental period (Fig. 2). In those rats, extracellular 2,3-DHBA was higher, though not with statistical significance, than that in animals given active AAO alone during the periods when the remarkable increase in extracellular 2,3-DHBA appeared in the rats given CO plus inactivated AAO (Fig. 2). In the previous study (Hara et al., 2009), the difference in extracellular 2,3-DHBA between the groups treated with active and inactivated AAO alone was statistically significant by Student's *t*-test. However, the difference was not significant in the present study, where the data were analyzed for four groups treated with active or inactivated AAO alone and in combination with CO exposure by using one-way ANOVA followed by the Tukey–Kramer test. Taking these findings into account, we further analyzed the interaction between the effects of CO exposure and the intrastriatal administration of active AAO on extracellular 2,3-DHBA by using two-way ANOVA; the results indicated that the interaction was significant during the last 20-min period of CO exposure and the following two 20-min periods after termination of CO exposure (Fig. 2).

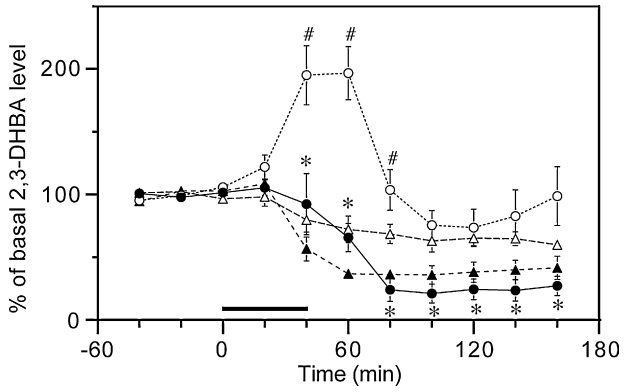

Fig. 2. Effect of active and inactivated AAO on the CO-induced increase in extracellular 2,3-DHBA in rat striatum. The solid horizontal bar indicates CO exposure at 3000 ppm and AAO administration for 40 min. Each symbol (△, inactivated AAO alone ($n = 6$); ▲, active AAO alone ($n = 6$); ○, inactivated AAO + CO ($n = 5$); ●, active AAO + CO ($n = 5$)) with a vertical bar indicates the mean ± SEM. The basal extracellular 2,3-DHBA was 0.0591 ± 0.0038 pmol/40 μL/20 min ($n = 22$). Significant difference ($p < 0.05$) by one-way ANOVA followed by Tukey–Kramer test; *inactivated AAO + CO vs. active AAO + CO; #inactivate AAO alone vs. inactivated AAO + CO. Interaction between active AAO and CO by two-way ANOVA; $F(1,18) = 5.161$ ($p < 0.05$) at 40 min; $F(1,18) = 14.827$ ($p < 0.01$) at 60 min; $F(1,18) = 5.700$ ($p < 0.05$) at 80 min.

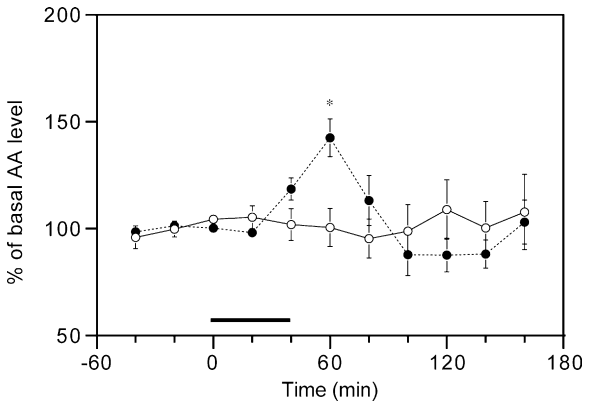

Fig. 3. Effect of CO exposure on extracellular AA in rat striatum. The solid horizontal bar indicates CO exposure at 3000 ppm for 40 min. Each symbol (○, air; ●, CO) with a vertical bar indicates the mean ± SEM obtained from five rats. The basal extracellular AA was 341.6 ± 39.9 pmol/40 μL/20 min ($n = 10$). *Significant difference ($p < 0.05$) between air and CO by Student's *t*-test.

3.2. Effect of CO exposure on extracellular AA in rat striatum

Fig. 3 showed the changes in extracellular AA in the striatum of rats exposed to 3000 ppm CO. Extracellular AA began to increase during the last 20-min period of the CO exposure, reaching a statistically significantly higher level, as compared with that of the control (room air alone) during the 20-min period immediately after the termination of CO exposure. Thereafter, it returned to the control level.

3.3. Effect of DFO on the CO-induced increase in extracellular 2,3-DHBA

As shown in Fig. 4(A), the basal level of 2,3-DHBA was significantly decreased by perfusion in the presence of 1 mM DFO, as previously reported (Hara et al., 2009). Under such conditions, DFO completely abolished the increase in 2,3-DHBA induced by CO exposure (Fig. 4(B)).

3.4. Effect of CO exposure on extracellular chelatable iron in rat striatum

In the present method for chelatable iron, the peak area for the perfusate of rat striatum before CO exposure was larger than that for the corresponding perfusing solution. However, the extracellular chelatable iron level was below the detection limit (2 pmol/40 μL), and did not reach the detectable level in rats exposed to 3000 ppm CO in room air ($n = 4$) or to room air alone ($n = 4$) (data not shown).

4. Discussion

In the present study, we first examined the effect of intrastriatal administration of AAO on •OH generation in the striatum of rats exposed to 3000 ppm CO, since direct administration of AAO, which rapidly degrades extracellular AA (Schenk et al., 1982), into the striatum is used to explore the role of AA in this brain region in vivo (Brazell and Marsden, 1982; Rebec and Wang, 2001). In rats treated with CO in combination with inactivated AAO, •OH generation in terms of extracellular 2,3-DHBA was remarkably stimulated in the striatum, as previously seen in rats treated with CO alone

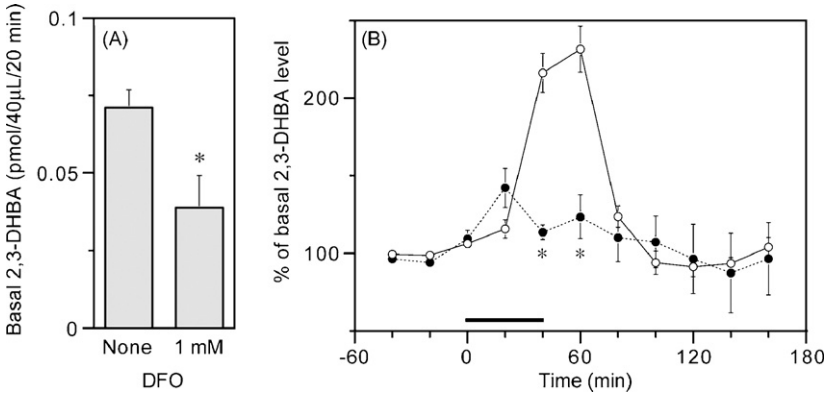

Fig. 4. Effect of DFO on the basal extracellular 2,3-DHBA (A) and the CO-induced increase in extracellular 2,3-DHBA (B) in rat striatum. DFO (1 mM) was dissolved in the perfusing solution and administered throughout the experimental period. Each column and symbol (○, 0 mM DFO; ●, 1 mM DFO) with the vertical bar in (A) and (B), respectively, indicates the mean ± SEM obtained from five rats. The solid horizontal bar in (B) indicates CO exposure at 3000 ppm for 40 min. *Significant difference ($p < 0.05$) between 0 mM and 1 mM DFO by Student's *t*-test.

(Hara et al., 2004). Such stimulation of •OH generation disappeared in rats treated with CO in combination with active AAO. These findings suggest that a decrease in extracellular AA might result in suppression of •OH generation. This is confirmed by the statistically significant interaction between the effects of CO exposure and intrastriatal active AAO on •OH generation. It has been shown that the extracellular AA level in the striatum rapidly returns to the constant level after termination of direct AA administration at various doses into the striatum (Miele and Fillenz, 1996), and the existence of a control system that strictly regulates the extracellular AA level in the striatum in vivo has been proposed (Rice, 2000). This control system would compensate for the decrease of extracellular AA due to active AAO by releasing intracellular AA, leading to a lack of intracellular AA (Schenk et al., 1982). The present study showed that such conditions did not stimulate, but rather suppressed, •OH generation in rat striatum. In addition, direct administration of AA (sodium salt) into the striatum immediately stimulates •OH generation in a dose-dependent manner, and this stimulation disappears shortly after termination of the AA administration (Hara et al., 2009). Taking these findings together, CO poisoning might cause an increase in extracellular AA, leading to stimulation of •OH generation in rat striatum. In fact, CO exposure evoked a significant increase in extracellular AA in the striatum. However, the time-course of the CO-induced changes in extracellular AA was not completely superimposable on that of •OH generation; namely, although CO exposure remarkably stimulated •OH generation in rat striatum during the period of the last 20 min of CO exposure and the following 20-min period (immediately after termination of CO exposure), the significant increase in extracellular AA corresponded only to the second period of •OH generation. This suggests that other factors in addition to extracellular AA might be involved in the CO-induced •OH generation. Furthermore, the mechanism of •OH generation induced by CO exposure might be different between the two periods. On the other hand, although it has been proposed that AA is released to the extracellular space by a heteroexchange uptake mechanism involving increased extracellular Glu (Rice, 2000), the CO-induced increase in extracellular AA only partly corresponded to that in extracellular Glu observed in the previous study (Hara et al., 2004), suggesting that further study of the mechanism of the CO-induced increase in extracellular AA is still needed.

It has been shown that the brain contains non-heme iron, which is in a chelatable low-molecular-mass form (Gutteridge, 1992). Such iron is a promoting factor, in addition to endogenous AA, of the lipid peroxidation reaction of brain tissues in vitro (Sharma and Krishna Murti, 1976; Zaleska and Floyd, 1985). This implies that •OH may be generated through the Fenton reaction, which is accelerated by redox cycling of chelatable iron associated with AA (Carr and Frei, 1999; Duarte and Lunec, 2005). Direct administration of either $FeCl_2$ (Triggs and Willmore, 1984) or AA (Hara et al., 2009) to rat brain with normal levels of AA and chelatable iron enhances oxidative stress. Thus, an increase of either extracellular AA or chelatable iron, or both, could stimulate •OH generation in the brain in vivo. This may be supported by the strong suppression of •OH generation, associated with an increase in extracellular AA, by an iron chelator, DFO, as well as by active AAO, in rat striatum (Hara et al., 2009). In the present study, the CO-induced •OH generation in rat striatum was completely abolished in the presence of DFO, suggesting that chelatable iron is the most likely candidate for the above factor participating in the CO-induced •OH generation. It is noteworthy that MRI revealed iron deposition in various brain regions, including the striatum, in patients following acute CO poisoning (Chang et al., 1992) and severe ischemic–anoxic insults (Dietrich and Bradley, 1988). Nilsson et al. (2002) successfully determined extracellular chelatable iron in several regions of newborn pig brain by means of microdialysis in combination with a newly developed method using an iron chelator, BPS, and reported an increase in extracellular chelatable iron in the striatum and white matter due to hypoxic hypoxia. We improved their method to make it more sensitive and examined the effect of CO exposure on extracellular chelatable iron in rat striatum. The results indicated the existence of chelatable iron in the extracellular space of rat striatum, but its level was too low to quantify. Therefore, the effect of CO exposure on extracellular chelatable iron remains unclear.

In conclusion, profound suppression of CO-induced •OH generation by active AAO and DFO strongly suggests that both extracellular AA and chelatable iron might play key roles in •OH generation in rat striatum due to CO poisoning, in which the prooxidant nature of AA might be elicited in the extracellular space. The changes in extracellular AA are consistent with at least a partial role in CO-induced •OH generation. Studies of how iron participates in the •OH generation may help to uncover the precise mechanism of CO toxicity to the brain.

Conflicts of interest

None.

Acknowledgments

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