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# Heme oxygenase–carbon monoxide pathway is involved in regulation of respiration in medullary slice of neonatal rats

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**Abstract**

Carbon monoxide (CO) is a novel biological messenger molecule. It is well known that CO can be synthesized in mammalian cells. In addition, CO is also demonstrated to participate in many physiological processes, such as vasomotion, thermoregulation and respiratory regulation. The purpose of our present study was to investigate the role of heme oxygenase–carbon monoxide (HO–CO) pathway in central regulation of respiration. The experiments were carried out on the medullary slices of neonatal Sprague–Dawley rats. The discharge activity of the hypoglossal rootlets was recorded to indicate the central rhythmic respiratory activity and its duration (DD), interval (DI), frequency (DF) and integrated amplitude (IA) were analyzed. The slices were perfused with ZnPP-9 (a potent inhibitor of heme oxygenase), CO and hemin (substrate of heme oxygenase), respectively, to observe their effects on respiratory activity. The results obtained were as follows: ZnPP-9 could decrease DD, DI and IA, and increase DF ($P<0.05$); exogenous CO caused a decrease in DD and DF, and an increase in DI and IA ($P<0.05$); in response to hemin, DI and IA decreased, DF increased ($P<0.05$), and DD did not change significantly ($P>0.05$); administration of both ZnPP-9 and hemin could decrease DI, and increase DF ($P<0.05$), but did not affect DD and IA significantly ($P>0.05$). It can be concluded from the results above that the HO–CO pathway may be involved in the regulation of rhythmic respiration at the level of medulla oblongata.


*Keywords:* Carbon monoxide; Heme oxygenase; Respiration; *In vitro*; Neonatal rat

Carbon monoxide (CO) has been thought to be noxious and harmful to the body for a long time. More than five decades ago, Sjostrand [18] suggested that CO is formed during the breakdown of heme in the human body. In 1981, Meyer [14] reported that CO appeared to interact with iron–sulfur centers of a variety of enzymes and then to inhibit the enzymes. In 1986, two forms of heme oxygenase (HO) were characterized from rat liver [9]. In the hematological system, CO had been shown to inhibit platelet aggregation apparently due to activation of soluble guanylyl cyclase (sGC) [1]. Based on these investigations, Marks et al. [10] presumed that CO may have physiological roles. By *in situ* hybridization, Verma et al. [20] demonstrated a discrete neuronal localization of mRNA for the constitutive form of HO throughout the brain. Besides, in primary cultures of olfactory neurons, zinc protoporphyrin-9 (ZnPP-9), a potent selective inhibitor of HO, depletes endogenous cyclic guanosine 3′, 5′-monophosphate (cGMP). Thus, CO, like nitric oxide, was believed to be a new physiological regulator of cGMP. These findings proved the presumption of Marks and suggested that CO may function as a neurotransmitter. Now, it is well known that CO can be synthesized in mammalian cells. In addition, CO has also been demonstrated to participate in many physiological processes, such as vasomotion [7], thermoregulation [4] and respiratory regulation [16].

Endogenous CO arises from the cleavage of the heme molecule yielding biliverdin, free iron and CO, a process catalyzed by HO. Some metalloporphyrins, such as ZnPP-9, SnPP-9 and ZnDPBG, are selective inhibitors of HO and can reduce formation of endogenous CO. Three distinct HO isoforms encoded by different genes have been identified to date, inducible HO-1 [9], constitutive HO-2 [17] and constitutive HO-3 [13], among which HO-1 and HO-2 are the most studied.

Expression of HO-2 mRNA was seen in the rostral ventrolateral medulla (RVLM) under both control and hypoxic conditions, whereas expression of HO-1 mRNA was only seen in the RVLM by induction of hypoxia. HO-2 was immunocyto-

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chemically localized in the RVLM to the pre-Bötzinger complex (PBC) which has been considered to be critical for the genesis of rhythmic respiration [12]. Electrophysiological studies on neurons dissociated from the RVLM showed that, in response to SnPP-9, many of these neurons increased baseline-firing frequency that is associated with depolarization of the membrane [11]. These observations indicate that the HO–CO pathway may be involved in central regulation of respiration.

The present study was carried out to investigate the possible roles of HO–CO pathway in central regulation of respiratory activity on neonatal rats *in vitro*.

The experiments were performed on medullary slices from either male or female neonatal (0–3 days) Sprague–Dawley rats. All procedures were reviewed and approved by Sichuan University Committee on the Use of Live Animals in Research, and conformed to the Principles of Laboratory Animal Care (NIH publication no. 86-23 revised 1985). The animals were anesthetized with ether by inhalation and decapitated, and the isolated brainstem was placed in the slicing chamber which was filled with ice-cold artificial cerebrospinal fluid (ACSF) bubbled with carbogen (95% $O_2$ and 5% $CO_2$). The ACSF contained (in mmol/L): 129 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 21 $NaHCO_3$, 1 $KH_2PO_4$, and 30 D-glucose, pH 7.4. The brainstem was positioned with the dorsal side upward, facing to the slicing blade at 20° to the rostral end of the block. In ice-cold ACSF, a single transverse slice of 1000–1500 μm thick was prepared, which was at the level of around the obex and contained functional respiratory neuronal network. Then the slices were transferred to a recording chamber and continuously perfused with oxygenated ACSF at a rate of 4–6 mL/min at 29 °C, pH7.4. To obtain and maintain consistent respiratory rhythmic activity, the KCl concentration of the perfusing ACSF was raised from 3 to 7 mmol/L (meanwhile the concentration of NaCl was reduced from 129 to 125 mmol/L to balance the osmotic pressure). The slices were incubated for 30 min before starting the experiments.

Glass suction electrodes filled with ACSF were used to record the rhythmic respiratory activity from the cut ends of the hypoglossal rootlets (Fig. 1A). Signals were amplified, filtered ($\tau = 0.001$ s, $F = 1$ kHz), and integrated with a time constant of 50 ms by BL-420E$^+$ biological signal processing system (Taimeng Biotech. Co., China). The discharge duration (DD, Fig. 1B), discharge interval (DI, Fig. 1B), discharge frequency (DF, number of discharge in 1 min) and integrated amplitude (IA, Fig. 1B) of hypoglossal rootlets were analyzed.

The slices were divided into five groups ($n = 8$ for each group): control, ZnPP-9, exogenous CO, hemin and ZnPP-9 + hemin. In the control group, the slices were perfused with ACSF during the whole process. In the ZnPP-9 group, the slices were perfused with ZnPP-9-ACSF (20 μmol/L) for 13 min. In the exogenous CO group, ACSF was bubbled with CO for 15 min just before the perfusion, then the slices were perfused with CO-ACSF [16] for 8 min. In the hemin group, the slices were perfused with hemin-ACSF (50 μmol/L) for 8 min. In the ZnPP-9 + hemin group, the slices were perfused with ZnPP-9-ACSF for 5 min, then with hemin-ZnPP-9-ACSF for 8 min; the concentrations of ZnPP-9 and hemin were the same as the ZnPP-9 group and the hemin group. The discharge activity of hypoglossal rootlets before chemical application was recorded for 5 min as baseline of the activity for each group. From the end of administration of chemicals, the slices were continuously perfused with ACSF for washout.

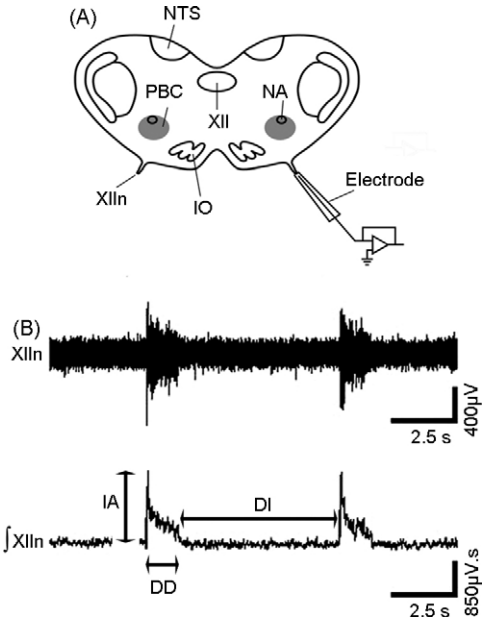


Fig. 1. Schematic diagram of recording technique of hypoglossal rootlets burst of medullary slices of neonatal rats. (A) The transversal view of medullary slice. IO: inferior olivary nucleus; NA: nucleus ambiguous; NTS: nucleus tractus solitarii, PBC: pre-Bötzinger complex; XII: hypoglossal nucleus; XIIn: hypoglossal rootlets. (B) The burst activity of hypoglossal rootlets, raw recording (top) and integrated activity (bottom), respectively. DD: discharge duration; DI: discharge interval; IA: integrated amplitude of discharge.

All data were normalized according to the baseline. Normalized DD, DI, DF and IA of hypoglossal rootlets in each 5 min were reported as means ± S.E.M. and statistically analyzed with two-tail repeated measure ANOVA. Statistical analysis were performed by SPSS 13.0 for Windows; $P$ values of <0.05 were considered statistically significant.

Each burst of hypoglossal rootlets had a rapid rising phase that was followed by a slowly decrementing period (Fig. 1B), which is similar to those published elsewhere [19]. Analysis of the rectified and integrated signal from medullary slices revealed that rhythmic activity was stable for more than 1 h and in some instances still could be recorded up to 8 h later. The activity of the rootlets perfused with ACSF during 45 min observing time was shown in Fig. 2.

ZnPP-9 can inhibit endogenous CO production through its inhibitory effect on HO. In the present experiments, ZnPP-9 was bath-applied continuously for 13 min at 20 μmol/L to investigate the possible role of the HO–CO pathway in central respiratory regulation. After administration of ZnPP-9, DD shortened by 6.65% ($P < 0.05$), DI shortened by ∼11.84% ($P < 0.05$), DF increased by ∼14.27% ($P < 0.05$), and IA decreased by ∼3.41% ($P < 0.05$), as shown in Fig. 3.

To further confirm the effects of CO, we investigated the effects of exogenous CO on discharge of hypoglossal rootlets of medullary slices. In response to exogenous CO, DD shortened by