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Effect of 2,4-dinitrophenol under non-oxygenated condition in pons–medulla–spinal cord preparations in newborn rats: Comparison with medulla–spinal cord preparations

Chikako Saiki *, Masaya Makino, Shigeji Matsumoto

Department of Physiology, Nippon Dental University, School of Life Dentistry at Tokyo, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan

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Abstract

We tested whether depression of respiratory frequency (fR) under non-oxygenated artificial cerebrospinal fluid (aCSF) in pons–medulla–spinal cord (PMS) and medulla–spinal cord (MS) preparations is significantly influenced by the mitochondrial uncoupler 2,4-dinitrophenol (2,4-DNP) in newborn rats.Preparations were obtained from 0- to 4-day-old rats, and fR was monitored at the C4 ventral root in environmental temperature (24 °C). 2,4-DNP was dissolved in aCSF (1, 10 or 30 μM; pH 7.4), and we measured fR in PMS ($n = 19$) and MS ($n = 16$), both of which were superfused with aCSF equilibrated with oxygenated (95% O_2–5% CO_2) or non-oxygenated (10% O_2–5% CO_2, balanced with pure N_2) gas.Our results showed that: (1) fR was significantly lower in PMS than MS, (2) fR was significantly decreased under non-oxygenated aCSF in both PMS and MS and (3) fR under non-oxygenated aCSF was significantly increased by 2,4-DNP applications at 10 and 30 μM in PMS but not in MS.Our results suggest that depression in fR under non-oxygenated aCSF in PMS and MS may not be due simply to O_2 limitation, and 2,4-DNP has a stimulant effect on the medullary respiratory rhythm generator (RRG) through pontine RRG regulatory mechanisms under non-oxygenated aCSF.

Keywords: Hypoxia; 2,4-Dinitrophenol; Uncoupler; Pons–medulla–spinal cord preparations; Medulla–spinal cord preparations; Respiratory frequency

In newborn rats, respiratory frequency (fR) is faster in medulla–spinal cord preparations (MS) than in pons–medulla–spinal cord preparations (PMS) [3,6,23] under oxygenated aCSF (95% O_2–5% CO_2). This difference in fR has been explained by early studies [3,7], in which it has been suggested that the pontine region may exert a tonic inhibitory effect on the respiratory rhythm generator (RRG), including Pre-I neurons (i.e. those included in the para-facial respiratory group) and the pre-Bötzinger complex, in the rostral ventrolateral medulla (RVLM) [1,10,18,21]. Moreover, although the tonic fR inhibition in PMS is proposed to be induced, at least in part, by activation of α_2-adrenergic receptors in the neurons within the RVLM via permanent release of noradrenaline (NA) from the pontine noradrenergic A5 area [3], exogenous NA application to PMS (in particular, to the pontine component) under oxygenated aCSF increases fR through its effect on the A5 neurons, which are though to be inhibited by exogenous NA [4]. These results suggest that pons does not only act to slow down the respiratory rhythm in the PMS compared to that in the MS, but also plays a part in the fine regulation of fR in the RRG, under oxygenated aCSF.

It has been demonstrated in PMS and MS in newborn rats that fR in these preparations decreases under non-oxygenated aCSF (8% O_2, 2% CO_2, balance N_2, pH 7.8), and it has been suggested that this decrease is due either to active inhibition of the medullary respiratory network or to direct inhibition of respiratory neurons by O_2 deprivation [17]. Although it has not been clarified whether different mechanisms of depression under non-oxygenated aCSF exist between PMS and MS, active inhibition of fR may exist, particularly in PMS. This is because an inhibitory chemosensitive network for hypoxia in the brainstem is thought to reside within the pons and thalamus [16]. Different O_2 demands for fR maintenance may also exist between PMS and MS: the lower fR for PMS could cause PMS to consume less O_2 comparing to MS, which would need more energy to maintain their higher fR.

* Corresponding author. Tel.: +81 3 3261 8740; fax: +81 3 3261 8740.
E-mail address: chikako@tky.ndu.ac.jp (C. Saiki).

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In earlier in vivo studies, pharmacological stimulation – for example, the systemic administration of a mitochondrial uncoupler, 2,4-dinitrophenol (2,4-DNP) – has reversed hypoxic metabolic depression and increased oxygen consumption in rats [20] and turtles [5] or stimulated ventilation (including respiratory frequency) in hypoxia in dogs [9]. These results suggest that 2,4-DNP is a useful tool for testing whether hypoxic depression reflects a systemic regulatory process or a limitation in cellular O_2 availability [5,20]. Therefore, to investigate whether different mechanisms of fR depression under non-oxygenated aCSF exist between PMS and MS, we applied 2,4-DNP to both preparations (PMS and MS) taken from newborn rats and subjected to non-oxygenated aCSF.

The newborn rats (0- to 4-day-old, Wistar, $n = 35$) were deeply anesthetized with ether, and decerebrated at the intercollicular level for PMS and just rostral to the anterior inferior cerebellar artery for MS [3,13,23]. The cerebellum was removed, and the spinal cord was transected at the C7–C8 level. The preparation was superfused at a rate of 3–5 mL/min in a 3 mL recording chamber with solution of the following compositions (mM): KCl, 3.0; NaCl, 128; $MgSO_4$, 1.0; $NaHCO_3$, 24; NaH_2PO_4, 0.5; $CaCl_2$, 1.5; D-glucose, 30, i.e. artificial cerebrospinal fluid (aCSF) [11,13], initially equilibrated with 95% O_2–5% CO_2 (oxygenated gas) at 24 °C, pH 7.4. The chamber temperature was continuously monitored as environmental temperature throughout the experiments, and controlled to 24 °C. The experiments were approved by the Animal Ethics Committee of Nippon Dental University, School of Life Dentistry at Tokyo. The preparation was placed with the ventral surface upward in the chamber as described previously [13], and to obtain respiratory rate (fR, min^{-1}), the dissected C4 ventral root was recorded with glass suction electrodes connected to amplifiers (DAM-50, World Precision Instruments Inc., FL, USA), in which the signals were amplified and band-pass filtered (0.3–3 kHz). Data were recorded on paper (Omniace 8100, NEC, Tokyo, Japan), and stored on a PC computer (eMac, Apple Computer Inc., Tokyo, Japan) using the interface (PowerLab®, ADInstruments Japan, Tokyo, Japan) at the sampling frequency of 10 kHz on each signal for subsequent data analysis.

2,4-Dinitrophenol (Wako Pure Chemical Industries Ltd., Osaka, Japan) as a mitochondrial uncoupler was dissolved in the aCSF at known concentrations (1, 10 and 30 μM, pH 7.4 at 24 °C), and applied to the preparation by superfusion through flow pipes placed over the chamber. The 2,4-DNP was applied three times in this order (1, 10 and 30 μM). Each concentration of 2,4-DNP (1, 10 or 30 μM) was applied for 5–7 min and interposed with the inflow of aCSF, which did not contain 2,4-DNP for approximately 10 min. To see the effect of 2,4-DNP on fR in PMS and MS, fR was monitored under oxygenated aCSF for 20–30 min to obtain control fR (100%), and, approximately 15 min later, 2,4-DNP was applied under oxygenated aCSF (oxygenated aCSF group). To see the effect of 2,4-DNP under non-oxygenated aCSF on fR in PMS and MS, perfused aCSF was switched from oxygenated to non-oxygenated aCSF, which was equilibrated with 10% O_2–5% CO_2 (balanced with pure N_2); fR was monitored under oxygenated aCSF for 20–30 min to obtain control fR (100%), and approximately 15 min after starting non-oxygenated aCSF 2,4-DNP was applied under non-oxygenated aCSF (non-oxygenated aCSF group). Experimental protocol was same for both experimental groups and for both PMS and MS preparations, and whole experiment was finished within 80–100 min in each preparation. All presented values are means ± S.E.M. Comparisons were made by one-way repeated-measures ANOVA followed by the Bonferroni t-test or two-tailed paired or unpaired t-tests as appropriate ($P < 0.05$). Mean values of fR were expressed in percentage of control in Figs. 1 and 3 but in cycle per minute in Table 1.

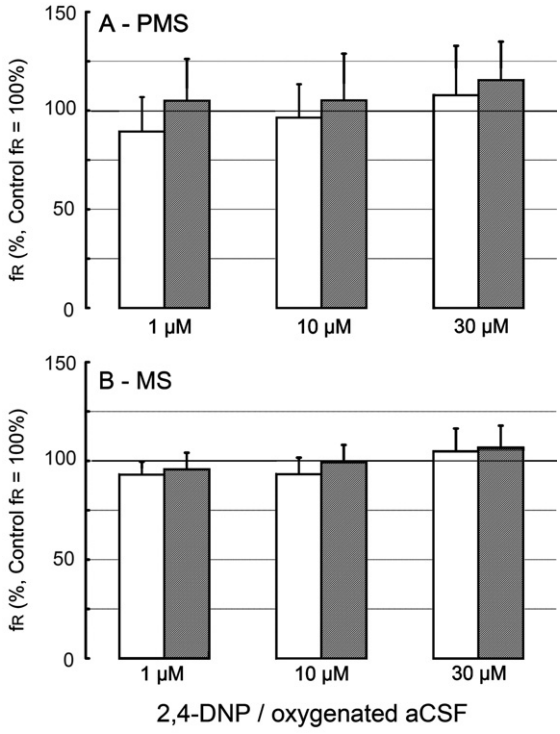

Fig. 1. Summary of fR under oxygenated aCSF (95% O_2–5% CO_2) before and during 2,4-DNP application (1, 10 or 30 μM) with and without pons. (A) Pons–medulla–spinal cord preparations (PMS, $n = 6$) and (B) medulla–spinal cord preparations (MS, $n = 6$). All values are presented as percentage of fR obtained under initial oxygenated aCSF (control fR, 100%) before further perfusion with oxygenated aCSF. Clear and solid bars, values obtained before and during each 2,4-DNP application, respectively. (A and B) Statistical analysis was performed between control (100%) and the value (%) obtained before or during each 2,4-DNP application ($^{*}P < 0.05$), and between the values (%) obtained before and during 2,4-DNP application at 1, 10 or 30 μM ($^{\S}P < 0.05$). No significant differences were found.

In the oxygenated aCSF group (Table 1), mean values of control fR and fR before each application of 2,4-DNP (1, 10 and 30 μM) were significantly lower in PMS ($n = 6$) than MS ($n = 6$). Histograms of Fig. 1 show mean fR in PMS and MS before (clear bars) and during (solid bars) 2,4-DNP application at 1, 10 and 30 μM. Note that 2,4-DNP applications never significantly affected fR in PMS and MS under oxygenated aCSF.

In the non-oxygenated aCSF group (Table 1), fR mean values were significantly lower in PMS ($n = 13$) than MS ($n = 10$). Fig. 2 shows recordings obtained in two PMS and MS preparations that were successively superfused with oxygenated aCSF and non-oxygenated aCSF. Switching from oxygenated aCSF to non-oxygenated aCSF reduced fR in both preparations. Thereafter,