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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

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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.

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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.

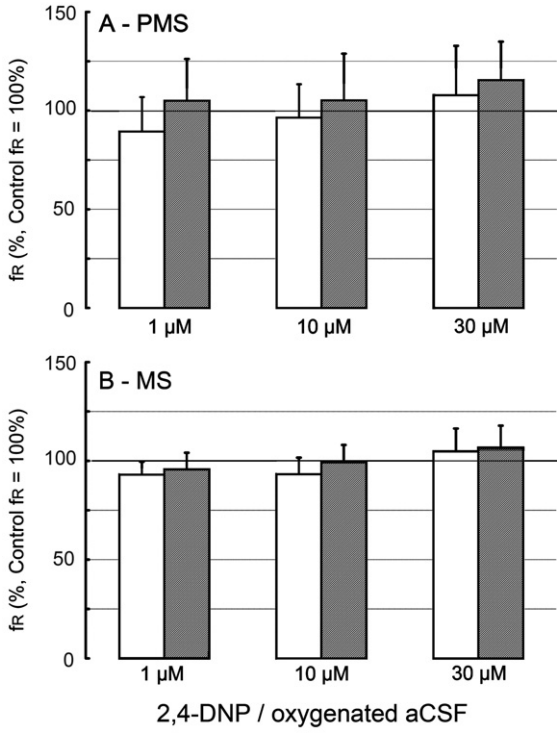

Fig. 1. Summary of fR under oxygenated aCSF (95% O_2–5% CO_2) before and during 2,4-DNP application (1, 10 or 30 μM) with and without pons. (A) Pons–medulla–spinal cord preparations (PMS, $n = 6$) and (B) medulla–spinal cord preparations (MS, $n = 6$). All values are presented as percentage of fR obtained 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,

Table 1
Respiratory frequency (fR) in oxygenated or non-oxygenated group under oxygenated aCSF (control), and before each application of 2,4-DNP (1, 10 or 30 μM) under oxygenated or non-oxygenated aCSF

Condition	Oxygenated aCSF (control)	Oxygenated or non-oxygenated aCSF/before application of 2,4-DNP		
		1 μM	10 μM	30 μM
Oxygenated aCSF group				
PMS ($n=6$)	1.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
MS ($n=6$)	4.0 ± 0.3¶	3.6 ± 0.3¶	3.6 ± 0.2¶	4.0 ± 0.1¶
Non-oxygenated aCSF group				
PMS ($n=13$)	1.8 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.2
MS ($n=10$)	5.3 ± 0.4¶	3.5 ± 0.4¶	2.9 ± 0.4¶	2.4 ± 0.3¶

Values (min^{-1}) are means ± S.E.M. Oxygenated aCSF group was treated with 2,4-DNP under oxygenated aCSF. Non-oxygenated group was treated with 2,4-DNP under non-oxygenated aCSF. PMS, pons–medulla–spinal cord preparations; MS, medulla–spinal cord preparations. In each oxygenated or non-oxygenated group, statistical analysis was performed between PMS and MS under each experimental condition.
¶ $P<0.05$.

applying non-oxygenated aCSF containing 2,4-DNP (30 μM) did not significantly affect fR in MS but significantly increased it in PMS. Histograms of Fig. 3 show mean fR in PMS and MS before (clear bars) and during (solid bars) application of non-oxygenated aCSF containing 2,4-DNP at 1, 10 and 30 μM.

Our results showed that the initial fR under control oxygenated aCSF was always lower in PMS than in MS, as reported by earlier studies [3,6,13,23]. When we exposed these preparations to non-oxygenated aCSF, we found significant fR depression in both PMS and MS (Fig. 2), a result compatible with those of an earlier study [17], and observed that fR in PMS was lower than that in MS before 2,4-DNP application, as seen under control oxygenated aCSF (Table 1).

Since this study could be the first study for effects of 2,4-DNP on the respiratory rhythm in the brainstem–spinal cord preparation, we first examined its effects under oxygenated aCSF. As preliminary experiments showed that prolonged applications of 2,4-DNP to PMS and MS (20 min, data not shown) had no effects on fR, we used repeated, short lasting applications. This led to long lasting experiments where resting fR gradually decreased while time elapsed (Table 1). Because we did not observe such gradual decrease in fR (percentage of control fR) during prolonged hypoxia (up to 60 min) without 2,4-DNP in preliminary experiments in MS (data not shown), it is possible that 2,4-DNP treatment has contributed to this gradual decrease in MS. Therefore, to find functional differences between PMS and MS under non-oxygenated aCSF, experimental protocol (example doses and duration of 2,4-DNP application and duration of oxygenated and non-oxygenated aCSF perfusion) was kept identical

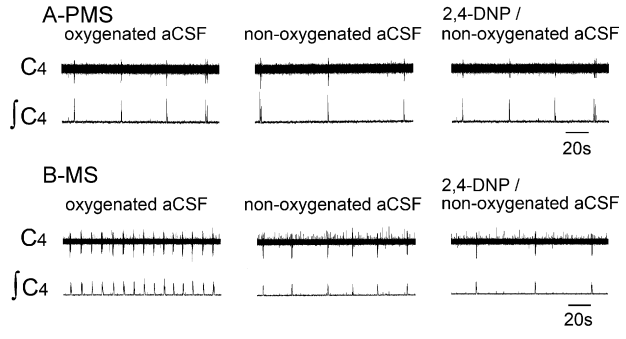

Fig. 2. fR under oxygenated aCSF, non-oxygenated aCSF and non-oxygenated aCSF plus 2,4-DNP (30 μM). (A) Examples in pons–medulla–spinal cord preparations (PMS). (B) Examples in medulla–spinal cord preparations (MS). *Abbreviations*: C4, fourth cervical spinal ventral root; ∫C4, integrated C4 activity; oxygenated aCSF, 95% O_2–5% CO_2 control conditions before non-oxygenated aCSF perfusion; non-oxygenated aCSF, 10% O_2–5% CO_2 (balanced with pure N_2) just before application of 30 μM 2,4-DNP; 2,4-DNP/non-oxygenated aCSF, 30 μM 2,4-DNP application under non-oxygenated aCSF.

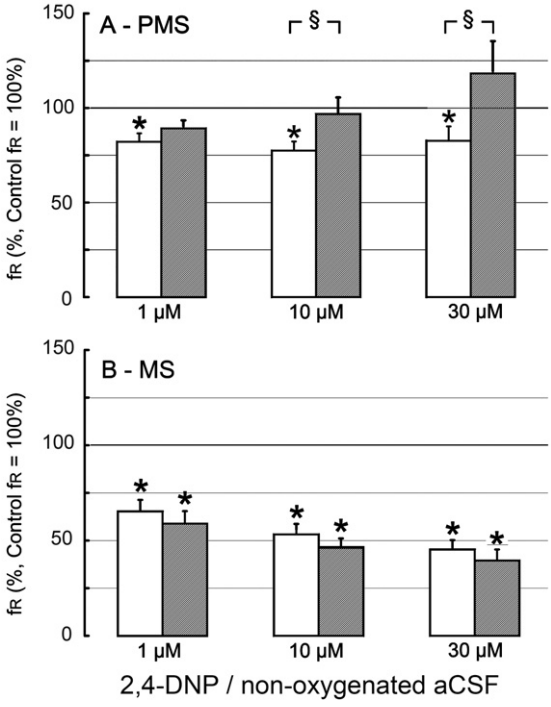

Fig. 3. Summary of fR under non-oxygenated aCSF (10% O_2–5% CO_2, balanced with pure N_2) before and during 2,4-DNP application (1, 10 or 30 μM) with and without pons. (A) Pons–medulla–spinal cord preparations (PMS, $n=13$) and (B) medulla–spinal cord preparations (MS, $n=10$). All values are presented as percentage of the fR obtained under initial oxygenated aCSF before non-oxygenated aCSF perfusion (control fR, 100%). Clear and solid bars, respectively, show values obtained before and during each 2,4-DNP application (1, 10 or 30 μM). (A and B) Statistical analysis was performed between control (100%) and the value (%) obtained before or during each 2,4-DNP application ($^{*}P<0.05$) or between the values (%) obtained before and during 2,4-DNP application at 1, 10 or 30 μM ($^{§}P<0.05$).

for PMS and MS. In addition, because we performed our experiments at slightly lower temperature (i.e. 24 °C) than that used in the most of studies from other laboratories [3,4,6,11,17,18,23], we cannot neglect a possibility that less apparent fR depression under non-oxygenated aCSF in PMS compared to MS and insignificant effect of 2,4-DNP on fR in MS compared to PMS (Fig. 3) are due to the different temperature sensitivity between PMS and MS. For example, earlier studies have shown that effect of drug application (example morphine) on fR in MS was less apparent at lower temperature (i.e. 22.5 and 25.5 °C) than that obtained at higher temperature (i.e. 28.5 and 31.5 °C) [22], whereas other drug application (example noradrenaline) in PMS significantly increased fR at 23 °C as seen at 27 °C [13]. In addition, in vivo study on cats, focal cooling of the intermediate area of ventral medullary surface to 20 °C attenuated the ventilatory augmentation caused by 2,4-DNP [19]. Although further investigations are needed to clarify possible effects of 2,4-DNP and temperature on fR in brainstem–spinal cord preparations, significant effect of 2,4-DNP under non-oxygenated aCSF (Figs. 2 and 3) suggests that fR depression under non-oxygenated aCSF is not simply due to O_2 limitation, at least in PMS at a fixed temperature (i.e. 24 °C), and that pons may play a part in fine regulation of fR in the RRG in the RVLM under non-oxygenated aCSF.

2,4-DNP is one of uncoupling agents, which are known to allow electron transport in mitochondria but to prevent the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) by uncoupling the essential linkage between electron transport and ATP synthesis. In the presence of 2,4-DNP, the free energy released by electron transport appears as heat rather than as newly made ATP, and the efficiency of ATP production is decreased [12]. In fact, when 2,4-DNP was applied to in vivo animals, it stimulated oxygen consumption even in hypoxia [5,20] and stimulated ventilation, as occurs in exercise [9]. Furthermore, the stimulant effect on metabolism is not limited to peripheral organs, and it has been demonstrated in vitro to stimulate cellular respiration in cerebral tissues in newborn rats [8], even under hypoxia [14]. Nevertheless, because the mechanisms of 2,4-DNP action, apart from by metabolic stimulation, remain unclear, we cannot explain fR increase at application of 2,4-DNP in PMS under non-oxygenated aCSF simply by its effect on metabolism (Figs. 2 and 3). For example, 2,4-DNP may cause decrease in cellular ATP content and the resulting defect of many cellular functions including active transport, in particular, in glucose- and O_2-limited in vitro experimental conditions.

Insignificant effect of 2,4-DNP under oxygenated aCSF (Fig. 1) further suggests that an increase in fR in PMS under non-oxygenated aCSF is not due to direct action of 2,4-DNP on the RRG, and that 2,4-DNP is acting only when it is applied to preparations retaining the pons (PMS) placed under non-oxygenated aCSF. This means that 2,4-DNP is acting on some pontine structures that: (1) modulate the medullary RRG and (2) are sensitive to hypoxia. Recently, it has been suggested that genetic factors are important for the prenatal maturation of the respiratory network in mammals. For example, Ret gene is known to encode a transmembrane tyrosine kinase receptor, and Ret-null mutant mice fetuses have been reported to have reduced NA contents in the pons (but not in medulla) and reduced number of pontine A5 and A6 neurons (but not in medulla), to show abnormal response to central hypoxia and NA application [24]. Therefore, it is possible that 2,4-DNP may enhance release of endogenous NA in PMS and modulate the RRG though pontine A5 and A6 adrenergic neurons [7] under hypoxic conditions. This hypothesis is not unlikely, because catecholamine is known to release in response to a stressor, such as acute hypoxia [15], and, for example, in isolated adult rat tail artery, 2,4-DNP has been reported to enhance stimulation-evoked NA release [2].

In conclusion, the fact that administration of 2,4-DNP (10 or 30 μM) in PMS under non-oxygenated aCSF restored the fR toward control levels (Figs. 2 and 3) suggests that the mechanisms of fR regulation differ between PMS and MS, and that, under non-oxygenated aCSF, 2,4-DNP has a stimulant effect on the medullary RRG through pontine RRG regulatory mechanisms. Indeed, 2,4-DNP application increased fR in preparations retaining the pons (PMS) but not in preparations lacking the pons (MS).

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