Experimental and Molecular Pathology 101 (2016) 157–162

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Creatine and creatine pyruvate reduce hypoxia-induced effects on phrenic nerve activity in the juvenile mouse respiratory system

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ARTICLE INFO

Article history:
Received 21 January 2016
and in revised form 29 May 2016
Accepted 19 July 2016
Available online 20 July 2016

Keywords:
Hypoxia
Creatine
Creatine pyruvate
Working heart-brainstem preparation

ABSTRACT

Adequate concentrations of ATP are required to preserve physiological cell functions and protect tissue from hypoxic damage. Decreased oxygen concentration results in ATP synthesis relying increasingly on the presence of phosphocreatine. The lack of ATP through hypoxic insult to neurons that generate or regulate respiratory function, would lead to the cessation of breathing (apnea). It is not clear whether creatine plays a role in maintaining respiratory phrenic nerve (PN) activity during hypoxic challenge.

The aim of the study was to test the effects of exogenously applied creatine or creatine pyruvate in maintaining PN induced respiratory rhythm against the deleterious effects of severe hypoxic insult using Working Heart-Brainstem (WHB) preparations of juvenile Swiss type mice. WHB's were perfused with control perfusate or perfusate containing either creatine [100 μM] or creatine pyruvate [100 μM] prior to hypoxic challenge and PN activity recorded throughout. Results showed that severe hypoxic challenge resulted in an initial transient increase in PN activity, followed by a reduction in that activity leading to respiratory apnea. The results demonstrated that perfusing the WHB preparation with creatine or creatine pyruvate, significantly reduced the onset of apnea compared to control conditions, with creatine pyruvate being the more effective substance. Overall, creatine and creatine pyruvate each produced time-dependent degrees of protection against severe hypoxic-induced disturbances of PN activity. The underlying protective mechanisms are unknown and need further investigations.

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1. Introduction

Cells require an adequate and continuous supply of the nucleoside triphosphate – ATP, to maintain the cell's physiological functions, such as preserving ionic gradients across cells and thus membrane potential in excitable cells. The majority of ATP is formed through oxidative phosphorylation and requires a good supply of oxygen to the cells. A decrease of arterial and tissue PO_2 during hypoxia has been shown to reduce ATP levels in brain cells (Erecińska and Silver, 2001).

Most of the ATP produced in neurons is consumed by ATP-driven pumps, such as the ubiquitous Na^+/K^+ ATPase, which maintains the resting membrane potential of the neurons. Due to the extraordinary number of neurons and glia cells in the brain, the Na^+/K^+ ATPases account for approximately 60% of total ATP consumption (Sahlin et al., 2015). Accordingly, depletion of cellular ATP results in the failure of the Na^+/K^+ ATPase and a consequent disruption of the membrane potential due to Na^+ leaking into the neurons and K^+, leaking out. As the leaked K^+ accumulates in the narrow interstitial space, it causes a progressive depolarization of neurons and glia cell membrane potential and disrupts functions (Ballanyi et al., 1996).

The respiratory neural network requires a constant supply of ATP to preserve proper rhythmic action potential (AP) discharges to bring about breathing patterns. Therefore, the preservation of neuronal ATP levels is crucial in maintaining the neuronal membrane potential and thus respiratory rhythm. Severe hypoxia of the respiratory neural network has been shown to provoke an initial increase in AP activity from that network, followed by a decrease in the AP activity, which could progress to a complete respiratory arrest (Prabhakar and Semenza, 2012). As long as the network has not entered a state of terminal apnea, these changes are normally reversible and the respiratory neural network function can be restored by re-oxygenation (Richter et al., 2008; Richter et al., 2000).

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http://dx.doi.org/10.1016/j.yexmp.2016.07.006
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It has been shown previously that exogenously applied creatine can enhance levels of phosphocreatine inside cells (Holtzman et al., 1989; Balestrino, 1995) and that the phosphocreatine can donate a phosphate group to ADP and, therefore, generate ATP to compensate for the reduced ATP synthesis. In hypoxic or anoxic conditions, neuronal ATP synthesis relies increasingly on the presence of phosphocreatine (Sahlin and Harris, 2011). In addition, creatine pyruvate, has been shown to act as an intermediate energy source in cells but there are limited published reports of creatine pyruvate's effects on nervous tissue (Dos Reis et al., 2013).

In the present study, mice Working Heart-Brain (WHB) preparations (Paton, 1996a; Paton, 1996b) were used to study the effects of severe hypoxic challenge on the activity of respiratory neurons, in particular PN action potentials. The PN activity was recorded from mice that were superfused with control solution, or a perfusate containing either creatine [100 μM] or creatine pyruvate [100 μM] prior to the hypoxic insult, to compare the effects that creatine or creatine pyruvate had on the results of the hypoxic shock to PN activity.

2. Methods

2.1. Working heart-brainstem (WHB) preparation

Analyses were performed on the *in situ* perfused WHB preparation (Paton, 1996a and 1996b) of Swiss mice obtained from existing institutional breeding colonies. The WHB preparation is an intra-arterially perfused preparation that has been shown to generate a normal rhythmic respiratory motor discharge pattern (Paton and Richter, 1995; Büsselberg et al., 2001; Büsselberg et al., 2003). The recording of PN action potentials in this system is an ideal model of respiratory activity, as the brainstem remains intact (Paton 1996a and 1996b) and thus possibly revealing any effects that creatine or creatine pyruvate may have on the action of respiratory neurons during hypoxia. The generation of spontaneous normal PN activity in the WHB preparation was taken as an indication of a functional preparation.

This preparation provides a more complete experimental system than brainstem alone, as it preserves the integrity of central coupling between the central respiratory rhythm generator and motor neurons that innervate the upper airways, respiratory pump muscles and heart.

All experimental procedures conformed to the recommendations of the European Commission (No L 358, ISSN 0378-6978), and protocols were approved by the Committee on Animal Research of the University of Göttingen. Every effort was made to reduce the number of animals used in this study and to minimize pain and discomfort of the animals used.

Mice (≥18 days) were deeply anaesthetized by placing the animals in a saturated atmosphere of halothane, until there was an absence of limb withdrawal reflexes following noxious pinching of a hind limb and the tail. During maintained anesthesia, the cortex, cerebellum and the pre-collicular brainstem were removed by aspiration. The animals were transected below the diaphragm, skin was removed and the upper body was transferred into ice-cooled, oxygenated artificial cerebrospinal fluid (ACSF) (mM: NaCl 125, KCl 5, KH_2PO_4 1.25, $CaCl_2$ 2.5, $MgSO_4$ 1.25, $NaHCO_3$ 25 and glucose 1.1) containing 2.1 g dextran/ 100 ml (to maintain oncotic pressure) which is bubbled with 95% O_2/ 5% CO_2 and pH adjusted to 7.4 with NaOH.

The WHB preparation was then transferred to a recording chamber, where the descending aorta was cannulated and perfused at a flow rate of 8–15 ml min^{-1} at 31 °C with Millipore-filtered, bubble-free ACSF. The perfusate had an osmolarity of 290 mosmol and was re-cycled after reoxygenation. Rhythmic contractions of respiratory muscles were evident within minutes following the onset of perfusion. Neuromuscular blockade, by addition to the perfusion solution of vecuronium bromide (3 mg ml^{-1}; Norcuron, Organon Teknika, Germany) was used to eliminate muscle movements, with no recorded effect on PN activity. The caudal end of the PN was drawn into a glass suction electrode (for details see Paton, 1996a and Paton, 1996b).

2.2. Electrophysiological recordings

Discharges were recorded from the left PN; the signals were passed through high- and low-pass filters and amplified with a differential amplifier in AC mode (methods are described in detail by Büsselberg et al., 2003). Cardiac action potentials, which were seen in the PN recordings, were electronically filtered from the recordings. All instrumentation for processing and amplification were constructed in-house. On line monitoring was accomplished with a digital oscilloscope (Tektronix, USA). Data was stored digitally for off-line analysis.

2.3. Drugs

Chemicals except creatine pyruvate were obtained from Sigma-Aldrich, Germany. Creatine pyruvate was purchased from Degussa, a division of Evonik Industries (Germany). Creatine and creatine pyruvate were delivered through the perfusion system at 100 μM concentration.

2.4. Experimental protocol

Control experiments: The WHB preparation was perfused with 95% O_2/5% CO_2 gassed ACSF solution for 30 min. The gas mixture was then replaced with 100% nitrogen (N_2) and the tissue subsequently perfused for 70 s with that solution. This replacement of the O_2 with N_2 resulted in a hypoxic challenge to the preparation. Throughout these changes the PN activity was recorded and the effects of hypoxic challenge on PN discharge activity, served as a control baseline.

To determine the viability over time of the experimental preparation, individual WHB's were perfused, for 60, 90 or 120 min, prior to the 70 s hypoxic challenge. The WHB preparation was then perfused with control O_2/CO_2 gassed ACSF to recover, during all of which the PN activity was recorded.

|Individual WHB preparations were also subjected to repeated hypoxic challenge to determine the viability of the system after this procedure. The control WHB preparations were subjected to 30 min perfusion with control ACSF followed by 70 s hypoxic ACSF challenge and then returned to perfusion with the control ACSF, during which the PN activity was recorded. Once PN activity had returned to initial levels *circa* 400 s after hypoxic challenge, the preparation was again exposed to a further hypoxic challenge and allowed to recover. This was repeated for a further 3 cycles (in total 5 hypoxic challenges per preparation).

Test substance Experiments: In these experiments a WHB preparation was perfused for 30, 60, 90 or 120 min with the O_2/CO_2 gassed control ACSF containing either creatine or creatine pyruvate prior to the 70 s hypoxic challenge with 100% N_2 gassed ACSF, which also contained either creatine or creatine pyruvate. As before, the PN activity was recorded form each preparation throughout the experiment.

2.5. Data analysis

The recorded instantaneous phrenic burst frequency was calculated as discharges per minute. Values of peak instantaneous frequency were converted to percentage (change from baseline) and presented as mean ± SEM. The significance between differences among groups was determined by analysis of variance (two-way ANOVA). To test for significance Fisher's *t*-test was performed considering $p < 0.05$ to be significant (*) and $p < 0.05$ as highly significant (**).

3. Results

PN activity could be recorded for a period in excess of 120 min from WHB preparations that were perfused with O_2/CO_2 gassed control ACSF

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