Prostaglandin E\textsubscript{2} differentially modulates the central control of eupnoea, sighs and gasping in mice

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Key points

- Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) augments distinct inspiratory motor patterns, generated within the preBötzinger complex (preBöC), in a dose-dependent way. The frequency of sighs and gasping are stimulated at low concentrations, while the frequency of eupnoea increases only at high concentrations.
- We used in vivo microinjections into the preBöC and in vitro isolated brainstem slice preparations to investigate the dose-dependent effects of PGE\textsubscript{2} on the preBöC activity.
- Synaptic measurements in whole cell voltage clamp recordings of inspiratory neurons revealed no changes in inhibitory or excitatory synaptic transmission in response to PGE\textsubscript{2} exposure.
- In current clamp recordings obtained from inspiratory neurons of the preBöC, we found an increase in the frequency and amplitude of bursting activity in neurons with intrinsic bursting properties after exposure to PGE\textsubscript{2}.
- Riluzole, a blocker of the persistent sodium current, abolished the effect of PGE\textsubscript{2} on sigh activity, while flufenamic acid, a blocker of the calcium-activated non-selective cation conductance, abolished the effect on eupnoeic activity caused by PGE\textsubscript{2}.

Abstract

Prostaglandins are important regulators of autonomic functions in the mammalian organism. Here we demonstrate in vivo that prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) can differentially increase the frequency of eupnoea (normal breathing) and sighs (augmented breaths) when injected into the preBötzinger complex (preBöC), a medullary area that is critical for breathing. Low concentrations of PGE\textsubscript{2} (100–300 nm) increased the sigh frequency, while higher concentrations (1–2 \textmu M) were required to increase the eupnoeic frequency. The concentration-dependent effects were similarly observed in the isolated preBöC. This in vitro preparation also revealed that riluzole, a blocker of the persistent sodium current (\textit{I_{NaP}}), abolished the modulatory effect on sighs, while flufenamic acid, an antagonist for the calcium-activated non-selective cation conductance (\textit{I_{CAN}}) abolished the effect of PGE\textsubscript{2} on fictive eupnoea at higher concentrations. At the cellular level PGE\textsubscript{2} significantly increased the amplitude and frequency of intrinsic bursting in inspiratory neurons. By contrast PGE\textsubscript{2} affected neither excitatory nor inhibitory synaptic transmission. We conclude that PGE\textsubscript{2} differentially modulates sigh, gasping and eupnoeic activity by differentially increasing \textit{I_{NaP}} and \textit{I_{CAN}} currents in preBöC neurons.

Detailed description of author contribution:
HK conceived the idea to study Prostaglandin E\textsubscript{2}
HK, AG3 and JMR designed the experiments
HK performed the majority of the experiments CC, FE, SZ and AG3 added somemmeasurements in brainstem slices
AD performed in vivo recordings in anesthetized animals
HK, CC and FE analyzed the data
HK and JMR wrote the paper
Introduction

Breathing has to adapt continuously and differentially to changes in the external and internal environment. At the centre of this adaptation lie neuronal networks that provide the flexibility to respond to ongoing changes. The preBötzinger complex (preBötC; Smith et al. 1991; Schwarzacher et al. 2011), a region that is essential for the generation of breathing (Ramirez et al. 1998; Wenninger et al. 2004; Tan et al. 2008), has been implicated in the modulatory response of breathing. This region, located within the ventrolateral medulla, is the target of several neuromodulatory systems (Doi & Ramirez, 2008, 2010; Koch et al. 2011; Ramirez et al. 2012). In addition to the classic neuromodulators such as noradrenaline (norepinephrine) (Ellenberger et al. 1990; Viemari & Ramirez, 2006; Zanella et al. 2014), substance P (Gray et al. 1999; Peña & Ramirez, 2004); acetylcholine (Shao et al. 2005) and serotonin (Peña & Ramirez, 2002; Ptak et al. 2009), this network is also regulated by molecules that can be activated through non-neuronal pathways. Some of these molecules are part of the inflammatory pathway and include prostaglandins, which are produced by cyclooxygenase-2 (COX-2) enzymes. Under physiological conditions, these inductive enzymes are expressed at basal levels in the brain. But a number of stimuli, including peripheral infections, pain, traumatic injury, hypoxia and hyperoxia, raise COX-2 expression and subsequently increase protein levels of prostaglandin E2 (Yamagata et al. 1993; Perez-Polo et al. 2011). PGE2, the major reaction product of the COX-2 enzymes, has been implicated in directly modulating the neuronal activity involved in several regulatory systems, including the regulation of pain (Ahmadi et al. 2002), sleep and wakefulness (Takemiyasawa, 2011), induction of fever (Scammell et al. 1996; Lazarus et al. 2007), synaptic plasticity and transmission (Akayama et al. 2006; Koch et al. 2010) and in the control of autonomic functions including respiration (Hofstetter et al. 2007).

Here we characterized the effects of PGE2 on the preBötC using in vitro slice preparations and in vivo preparations from freely breathing animals. Our data demonstrate that low concentrations (<300 nM) of PGE2 injected into the preBötC in vivo increased sigh frequency, but had no effect on normal breathing (eupnoea). Higher concentrations (1–2 μM) of PGE2 were required to also increase eupnoeic frequency. This response was mimicked in the preBötC isolated in an in vitro brainstem slice preparation. Thus our study contributes to the notion that PGE2 is an important modulator of respiratory activity. A comparison with existing studies (Ballanyi et al. 1997; Hofstetter et al. 2007) suggests that prostaglandins exert a variety of diverse effects that may result in adaptive and maladaptive responses of the respiratory network in health and disease.

Methods

All animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee at Seattle Children’s Research Institute. Mice were maintained with rodent diet and water available ad libitum in a vivarium with a 12 h light/dark cycle at 22°C.

In vivo recordings and microinjection into the preBötC

A total of six CD1 mice (postnatal days (P) 9–16) were anaesthetized with urethane (1.5 g kg\(^{-1}\)) by an intraperitoneal injection for in vivo measurements. At the end of the experiments animals were killed by transcardiac perfusion under terminal urethane anaesthesia. Mice, of either sex, were placed in a supine position, and the head was fixed with a stereotaxic apparatus. The neck of the mouse was opened from the ventral side, the trachea was cut, and plastic Y-shaped tubing for supplying oxygen was inserted into the proximal end of the trachea (cannulation). The bone of the skull covering the ventral brainstem was partially removed with small scissors and forceps. The dura and arachnoid membrane were removed to expose the ventral medulla. The surface of the ventral medulla was continuously perfused with 95% O\(_2\)–5% CO\(_2\)-equilibrated artificial cerebrospinal fluid (aCSF) solution at 30°C ± 0.5°C. In all cases, 100% oxygen was supplied through cannulation without artificial ventilation. The core body temperature during in vivo experiments was measured routinely in our laboratory and was stable at 36°C ± 1°C.
Electromyography recordings (EMG) of the intercostal muscles were recorded with a Teflon-covered Ag bipolar electrode. The skin over the abdominal and intercostal area on the right side was partially removed, and the bipolar electrode was placed on the surface of the intercostal muscles. Signals were AC amplified and bandpass filtered (8 Hz to 3 kHz).

Microsyringes (Hamilton microsyringe no. 80330, Hamilton, Reno, NV, USA) with 33 gauge needles containing PGE2 were positioned with a micro-manipulator for subsequent microinjections (KITE, World Precision Instruments, Sarasota, FL, USA). The needles of the microsyringes were inserted into the right preBöC from the ventral side. While constantly measuring intercostal EMG activity, PGE2 was injected into the right preBöC (0.3 μl min⁻¹). We did not attempt any bilateral injections to limit the damage to the preBöC, which would have compromised respiratory rhythm generation. As previously described and anatomically characterized, injections in the preBöC are routinely traced and localized to an area encompassing the preBötzinger area in close proximity to the nucleus ambiguus (Zanella et al. 2014). As also demonstrated in previous studies, the injections typically encompass an area that slightly exceeds the preBötzinger area, and the reader is referred to the study by Zanella et al. (2014) for further details.

The transverse slice preparation

Brainstem transverse slice preparations from CD1 (n = 65) mice of either sex (P0–15, Charles River Laboratories International, Inc., Wilmington, MA, USA) were obtained as also described in detail previously (Ramirez et al. 1996). All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Seattle Children’s Research Institute. Mice were deeply anaesthetized with isoflurane (4%) before quick decapitation. Isolated brainstems were then placed in ice-cold aCSF bubbled with carbogen (95% O₂–5% CO₂). The aCSF contained (in mM): 118 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 24 NaHCO₃, 0.5 NaH₂PO₄, and 30 D-glucose, pH 7.4. Brainstems were glued rostral end up onto an agar block for mounting into a vibratome (Leica Microsystems, GmbH, Wetzlar, Germany). A single 550–600 μm-thick slice was then taken. Slices were transferred into a recording chamber, continuously superfused with oxygenated aCSF, and maintained at a temperature of 30 ± 0.5°C. To initiate and maintain fictive respiratory rhythmic activity, the potassium concentration of the perfusate was raised from 3 to 8 mM over a period of 30 min. We routinely measured the PO₂ in the bath before, during and after the exposure to aCSF bubbled with 95% O₂ or 95% N₂, as described in detail in the study by Hill et al. (2011).

Extracellular population and intracellular current clamp recordings from the preBöC

In the transverse slice preparation, extracellular population recordings were obtained with suction electrodes positioned on the surface of the ventrolateral region containing the preBöC. To obtain a signal containing multi-unit action potential (AP) activity, extracellular signals were amplified 10,000-fold and filtered between 0.25 and 1.5 kHz using an AM instruments (A-M Systems, Sequim, WA, USA) extracellular amplifier (Fig. 1B, top trace). To facilitate the detection of bursts, this signal was rectified and integrated by using an electronic integrator with a time constant of 50 ms (Fig. 1B, middle trace) using home-built equipment. Intracellular current clamp recordings were obtained from respiratory neurons of the contralateral preBöC with the blind-patch technique. The patch electrodes were manufactured from filameted borosilicate glass tubes (Warner Instruments, Hamden, CT, USA 150TF), filled with a solution containing (in mM): 140 potassium gluconate, 1 CaCl₂, 6H₂O, 10 EGTA, 2 MgCl₂, 6H₂O, 4 Na₂ATP, and 10 Hepes (pH 7.2). In some cases the intracellular pipettes contained biocytin (4.5 mg ml⁻¹) to allow for the identification of neuron location and morphology. Recordings were low-pass filtered (0–2 kHz, Bessel 4-pole filter, ~3 dB). Neurons were identified as respiratory neurons by their discharge pattern in phase with the population activity of the contralateral preBöC (Fig. 1B, bottom trace).

Voltage clamp recordings of preBöC neurons

Whole cell patch clamp recordings of inspiratory neurons were obtained with a sample frequency of 10 kHz and a low-pass filter setting of 2 kHz. Recordings were made with unpolished patch electrodes, manufactured from borosilicate glass pipettes with a filament (Warner Instruments G150F-4, Warner Instruments, Hamden, CT, USA). The electrodes had a resistance of 3–5 MΩ when filled with the whole cell patch clamp pipette solution containing (in mM): 140 potassium gluconate, 1 CaCl₂, 6H₂O, 10 EGTA, 2 MgCl₂, 6H₂O, 4 Na₂ATP, and 10 Hepes (pH 7.2). The patch clamp experiments were performed with a patch clamp amplifier (Multipatch 700B, Molecular Devices, Sunnyvale, CA, USA), a digitizing interface (Digidata 1440A, Molecular Devices), and the software program pCLAMP 10.0 (Molecular Devices). Neurons located at least three to four cell layers (about 80–150 μm) caudal from the rostral surface of the slice were recorded under visual control. Neurons located directly at the slice surface were not examined because their dendritic processes were more likely to be damaged during the preparation than those of neurons located deeper within the slice. Current–response traces were recorded with either off- or online leak subtraction

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(P/4 protocol), eliminating the linear leak current and residual capacity currents. The 2 mV liquid junction potential was manually subtracted with the amplifier’s pipette offset regulator immediately before establishing the patch clamp configuration. The series resistance was always 80% compensated and regularly corrected throughout the experiments. We emphasize that whole cell voltage clamp recordings from neurons embedded in a functional network are accompanied by difficult space clamp control. This could lead to incorrect values for current amplitudes. Thus, recordings with obvious space clamp were discarded. Consulting equilibrium potential to holding potential relationships, all upward deflections had to be chloride-conducting, inhibitory postsynaptic currents (IPSCs), whereas all downward deflections had to be excitatory postsynaptic currents (EPSCs). EPSCs and IPSCs were analysed by using MiniAnalysis 5.41 (Synaptosoft, Inc., Decatur, GA, USA) and statistical analysis was performed with Prism (GraphPad Software, Inc., La Jolla, CA, USA). The synaptic drive current in individual neurons, which occurred in phase with the rhythmic population discharges of the preBötC, was assessed by low-pass filtering the intracellularly recorded current traces (10 Hz) and determining the negative peak amplitude (maximum negative current during the burst). This amplitude was then compared during control conditions in the presence of 100 nM PGE2 and in the presence of 2 μM PGE2 (Fig. 6Aa).

Results

PGE2 injections into the preBötC differentially affect sighs and eupneic breathing in vivo

In anaesthetized freely breathing CD1 mice (male and female, P9–16, n = 6) we tested the effect of microinjections of three different concentrations of PGE2 (100 nM, 300 nM and 1 μM) into the right preBötzinger complex. The breathing was recorded with EMG recordings from the intercostal muscles (Fig. 1A, for details see Methods). At the lower concentrations we observed an increase in sigh frequency (*P < 0.05, one-way ANOVA, Fig. 2A and B), while the eupneic frequency was unaltered (Fig. 2B). In contrast, higher concentrations of PGE2 (1 μM) led to a significant increase in sighs and eupnoea compared to control conditions (*P < 0.05, one-way ANOVA Fig. 2B).

PGE2 application directly modulates the activity in the preBötC

Next we tested the effects of PGE2 on the preBötC network in the isolated brainstem slice preparation of CD1 mice (male and female, P0–5). In a first set of extracellular experiments, the population activity of the preBötC was recorded with a surface electrode before and after bath application of PGE2. Similar to the in vivo experiments we found a dose-dependent effect of PGE2 on the fictive eupnoea and sigh activities generated within the slices. At low concentrations (10–100 nM) PGE2 strongly stimulated the frequency of sighs (Fig. 3A and B), without significantly affecting the amplitude, duration or frequency of eupnoea bursts generated in the preBötC (Fig. 3C). At higher concentrations (2 μM) PGE2 evoked a similar increase in sigh frequency as observed at lower concentrations, but PGE2 had an additional enhancing effect on the eupnoea frequency compared to control (Fig. 3C, **P < 0.01, paired t-test). The effects on both fictive eupnoea and fictive sighs were reversible upon washout.

PGE2 increases the fictive gasping response of the preBötC to hypoxia

To assess if PGE2 changes the activity of the preBötC during hypoxia, we quantified the response to severely reduced
levels of oxygen (95% N₂, 5% CO₂, Hill et al. 2011) in the absence and presence of different concentrations of PGE₂. For these experiments we applied PGE₂ for at least 10 min before the measurements. We characterized only one hypoxic exposure per slice. As previously described, exposure to hypoxia leads to a typical biphasic response with an early augmentation and a late depression phase in which the network reconfigures to generate fictive gasping (Fig. 4A, Telgkamp & Ramirez, 1999; Lieske et al. 2000; Peita et al. 2004; Hill et al. 2011). Slices exposed to moderate (100 nm, n = 6) or high (2 μM, n = 6) concentrations of PGE₂ (Fig. 4B, C and D) generated gasping activity with significantly increased amplitude and frequency values compared to untreated control slices (Fig. 4D, *P < 0.05, **P < 0.01, one-way ANOVA). Slices exposed to the low concentration (10 nm) did not show a significantly different gasping compared to control slices (n = 8, one-way ANOVA).

**Developmental changes in PGE₂ response**

To determine if the effect of PGE₂ was dependent on the age of the animals we tested a set of slices obtained from animals during their second postnatal week (P7–12, n = 6 for 2 μM, n = 4 for 10–100 nm) and compared them to the data of the slices obtained during the first 5 days of life (n = 6 for 2 μM, n = 14 for 10–100 nm). The increase of the sigh frequency induced by a bath application of a low or high concentration of PGE₂ was significantly more pronounced in slices of mice prepared during the first postnatal week (Fig. 5A, two-way ANOVA, Holm-Sidak’s multiple comparison test). By contrast, slices obtained during the second postnatal week revealed only a slight increase at the low concentration (10–100 nm) and a slightly more pronounced effect in response to the high concentration of PGE₂ (2 μM) (Fig. 5A). High concentrations of PGE₂ significantly increased the frequency of fictive eunpnea in both age groups and no significant difference between the first compared to the second postnatal week was detected (Fig. 5B).

**Spontaneous excitatory and inhibitory postsynaptic currents and the drive current**

To test if PGE₂ affects synaptic transmission between respiratory neurons we recorded from inspiratory preBötC neurons in voltage clamp to measure the excitatory and inhibitory synaptic events (spontaneous (s)EPSCs and (s)IPSCs). Inspiratory neurons were defined as neurons that received phasic input during the population activity measured with a surface electrode on the preBötC (Fig. 6Aa). We measured sEPSCs and sIPSCs between the rhythmic population bursts before and after adding PGE₂ (100 nm and 2 μM) to the bath (Fig. 6Ab and B). As described in detail in the Methods section, we quantified amplitude, decay time and frequency of the EPSCs and IPSCs as an average of 50 events for each condition (control, 100 nm PGE₂, 2 μM PGE₂, n = 5 for all groups). PGE₂ led to a small, but significant reduction of the amplitude of sEPSCs (*P < 0.05, one-way ANOVA) in the presence of 100 nm PGE₂, which returned to control values at the high concentration. All other measured parameters did not change when exposed to PGE₂ (Fig. 6B, one-way ANOVA). In addition, PGE₂ at high or low concentrations did not significantly change the drive current (Fig. 6Aa) compared to control conditions (Fig. 6B).

**PGE₂ increases intrinsic bursting activity in inspiratory neurons**

To test if PGE₂ modulates the intrinsic properties of preBötC neurons, we recorded from inspiratory neurons, which discharged in phase with the population burst recorded from the surface of the preBötC in the current clamp configuration (Fig. 7A). In a first set of experiments we tested the response of the neurons within the intact network (n = 9). Most neurons showed a slight depolarization of several millivolts in response to both tested concentrations (100 nm, +4.75 ± 3.10 mV, n = 4; 2 μM, +1.65 ± 1.53 mV, n = 5). However, there was no significant change in the drive potential underlying fictive eunpnea or fictive sighs (Fig. 7A). Next we tested

**Figure 2. Microinjections of PGE₂ into the preBötC increased the frequency of sighs and eunpneic breathing at different concentrations.**

*Figure 2. Microinjections of PGE₂ into the preBötC increased the frequency of sighs and eunpneic breathing at different concentrations. A, a typical example of the response to microinjections of PGE₂ (100 and 300 nm) into the preBötC of a freely breathing mouse. Note the increase of sighs at low concentrations (example trace in A and quantification in B). Higher concentrations (1 μM) of PGE₂ injections increased the frequency of eunpnea and sighs (*P < 0.05).*
the effect on the neurons in isolation from synaptic inputs. All examined neurons (n = 16) showed either spontaneous tonic firing or intrinsic bursting firing (Fig. 7B, n = 9), when pharmacologically isolated from fast synaptic inputs by the addition of 6-cyano-7-nitroquinoline-2,3-dione (CNQX; 20 μM), CPP (10 μM), picrotoxin (5 μM) and strychnine (1 μM) to the bath. Similar to the neurons tested with intact synaptic transmission, PGE₂ caused a slight depolarization (2.65 ± 3.00 mV, n = 16). The depolarizations caused by either 100 nM or 2 μM PGE₂ were not significantly different in the tonic firing cells or intrinsic bursting neurons.

In intrinsic bursting cells we found a significant increase in the amplitude and frequency of the bursting after exposure to 2 μM PGE₂ (Fig. 7C and D, *P < 0.05, paired t test, n = 9). It needs to be emphasized that bursting was irregular in some cases, and we also observed a substantial variability in the range of bursting in these cells. Thus, the evaluation of the burst frequency over a long period of time may not have been always consistent for all neurons. Previous studies identified two subtypes of pacemaker neurons based on the currents that are critical for the generation of the intrinsic bursts. Neurons that burst depending on the I_CAN were termed cadmium sensitive (CS), because bursting was blocked by cadmium, a blocker of calcium currents. Neurons with bursting properties that depend on the persistent sodium current were termed cadmium insensitive (CI) neurons, because their bursting persisted in the presence of cadmium (Thoby-Brisson & Ramirez, 2001; Peña et al. 2004). The stimulating effect of PGE₂ was found in CI pacemaker neurons (n = 4). These neurons were sensitive to 10 μM riluzole or insensitive to 200 μM Cd²⁺. A stimulating effect of PGE₂ was also found in CS neurons (n = 3). These neurons were sensitive to 200 μM Cd²⁺. In two neurons the response to Cd²⁺ or riluzole was not further tested. Since we only tested the high concentration of PGE₂, we were not able to distinguish from these experiments whether a specific cell type was essential for the specific effect of PGE₂ on sigh frequency. We therefore conducted additional extracellular experiments to assess the effect of these blockers at the network level.

**Differential contribution of I_CAN and I_NaP to the PGE₂ effect**

As mentioned above, it was previously shown that intrinsic bursting properties in the inspiratory neurons of the preBötC can depend either on the persistent sodium current (I_NaP) or on a calcium-activated non-selective cation conductance (I_CAN) (Peña et al. 2004). To test if I_CAN- or I_NaP-dependent bursting plays a critical role in