II-1β and prostaglandin E₂ attenuate the hypercapnic as well as the hypoxic respiratory response via prostaglandin E receptor type 3 in neonatal mice

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Siljehav V, Shvarev Y, Herlenius E. II-1β and prostaglandin E₂ attenuate the hypercapnic as well as the hypoxic respiratory response via prostaglandin E receptor type 3 in neonatal mice. J Appl Physiol 117: 1027–1036, 2014. First published September 11, 2014; doi:10.1152/japplphysiol.00542.2014.—Prostaglandin E₂ (PGE₂) serves as a critical mediator of hypoxia, infection, and apnea in term and preterm babies. We hypothesized that the prostaglandin E receptor type 3 (EP3R) is the receptor responsible for PGE₂-induced apneas. Plethysmographic recordings revealed that IL-1β (ip) attenuated the hypercapnic response in C57BL/6J wild-type (WT) but not in neonatal (P9) EP3R⁻/⁻ mice (P < 0.05). The hypercapnic responses in brain stem spinal cord en bloc preparations also differed depending on EP3R expression whereby the response was attenuated in EP3R⁻/⁻ preparations (P < 0.05). After severe hypoxic exposure in vivo, IL-1β prolonged time to autoresuscitation in WT but not in EP3R⁻/⁻ mice. Moreover, during severe hypoxic stress EP3R⁻/⁻ mice had an increased gasping duration (P < 0.01) as well as number of gasps (P < 0.01), irrespective of intraperitoneal treatment, compared with WT mice. Furthermore, EP3R⁻/⁻ mice exhibited longer hypneic breathing efforts when exposed to severe hypoxia (P < 0.01). This was then followed by a longer period of secondary apnea before autoresuscitation occurred in EP3R⁻/⁻ mice (P < 0.05). In vitro, EP3R⁻/⁻ brain stem spinal cord preparations had a prolonged respiratory burst activity during severe hypoxia accompanied by a prolonged neuronal arrest during recovery in oxygenated medium (P < 0.05). In conclusion, PGE₂ exerts its effects on respiration via EP3R activation that attenuates the respiratory response to hypercapnia as well as severe hypoxia. Modulation of the EP3R may serve as a potential therapeutic target for treatment of inflammatory and hypoxic-induced detrimental apneas and respiratory disorders in neonates.

prostaglandin E₂; EP3R; hypoxia; inflammation; hypercapnia

INFECTION during the neonatal period commonly induces hypoxic and potentially life-threatening apnea episodes (24). Apneas often precede episodes of sepsis and bacterial infection that are associated with sudden infant death syndrome (SIDS) (20, 54). SIDS and acute life threatening events (ALTE) are considered the result of inadequate arousal (16). Consequently, the ability to arouse might be the most important response during infarction as well as response to hypoxia and hypercapnia (41). Apneas commonly occur in premature infants. Although short respiratory pauses should be of little consequence when the infant is well oxygenated, recurring apneic pauses leading to chronic intermittent hypoxemia (CIH) yield long-term consequences such as a greater incidence of retinopathy of prematurity (8) and an increased risk for adverse neurological outcome (33). We have previously reported how the prostaglandin E₂ (PGE₂) pathway, induced by inflammation or hypoxia, leads to apneas and depresses infant respiration (25). We hypothesize that the prostaglandin E receptor type 3 (EP3R) is the main receptor mediating the deleterious effects of PGE₂.

PGE₂ release in the brain stem is the result of an inflammatory response to interleukin 1β (IL-1β), or a hypoxic event (25). IL-1β is produced during the acute-phase immune response to infection (7). It evokes a variety of sickness behaviors including respiratory depression (23, 39). A major and rapid pathway for IL-1β to act across the blood-brain barrier (BBB) is by binding to IL-1 receptors on vascular endothelial cells of the BBB, resulting in induction of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) activities (11, 25).

Following induction of COX-2 and mPGES-1 activity by proinflammatory stimuli such as IL-1β, PGE₂ is released into the brain parenchyma (11) and mediates several central effects including fever (5), pain (28), anorexia (12), and respiratory depression (51). PGE₂ depresses breathing in fetal and newborn sheep, mice, and in humans in vivo (25, 30, 51), and also inhibits respiration-related neurons in vitro (23, 25, 39). Others and we have suggested that PGE₂ serves as a critical mediator of inflammation and apnea (22, 24, 25, 48).

In addition to an inflammatory stimulus, hypoxia itself induces PGE₂ synthesis (31) that depresses respiration (43) and might lead to deleterious brain damage (32). During perinatal hypoxia PGE₂ metabolite levels in cerebrospinal fluid are increased and directly correlate with the severity of perinatal asphyxia in human neonates (3).

The role for PGE₂ as an intermediate messenger between circulating IL-1β and respiratory depression requires PGE₂ receptors in the brain stem, so-called EP receptors (EPR). EP2-4R receptors have been identified in the rat and mouse brain and brain stem (37, 49, 50). PGE₂ effects on breathing include decreased eupneic frequency, hypoxic response, and induction of apneas, and these are hypothesized to be mediated through EP3R in respiratory-related regions of the brain stem [e.g., nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM)] (10, 25).

Herein we addressed the role of EP3R in the respiratory response to IL-1β, hypercapnia, and hypoxia. The induced PGE₂ pathway may affect respiratory regulation in a detrimental way, and we need to further elucidate the underlying mechanism(s) to be able to respond with precise therapeutic interventions. In the present study we hypothesized that neonatal mice deficient in EP3R would have an altered respiratory response to hypercapnic and hypoxic challenge following inflammatory and hypoxia-induced PGE₂ release.

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Table 1. Respiratory parameters under basal conditions

<table>
<thead>
<tr>
<th></th>
<th>Weight</th>
<th>Fr, breaths/min</th>
<th>Vr, μl/g</th>
<th>Vv, μl.g⁻¹.min⁻¹</th>
<th>Tc, ms</th>
<th>Te, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT NaCl (n = 24)</td>
<td>4.4 ± 0.1</td>
<td>178 ± 6</td>
<td>5 ± 1</td>
<td>919 ± 155</td>
<td>37 ± 2</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>WT IL-1β (n = 25)</td>
<td>4.2 ± 0.1</td>
<td>182 ± 6</td>
<td>6 ± 1</td>
<td>1,135 ± 145</td>
<td>35 ± 2</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>EP3R⁻/⁻ NaCl (n = 21)</td>
<td>4.7 ± 0.2*</td>
<td>186 ± 6</td>
<td>5 ± 1</td>
<td>912 ± 159</td>
<td>37 ± 2</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>EP3R⁻/⁻ IL-1β (n = 20)</td>
<td>4.7 ± 0.2*</td>
<td>183 ± 7</td>
<td>5 ± 1</td>
<td>909 ± 183</td>
<td>38 ± 2</td>
<td>56 ± 4</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. WT, wild type; EP3R⁻/⁻, prostaglandin E receptor type 3 knockout. No differences were observed between strains or ip treatment for respiratory frequency (Fr), tidal volume (Vv), minute ventilation (Vv), inspiratory time (Tc), or expiratory time (Te). There was a difference in weight between genotypes, WT vs. EP3R⁻/⁻ (P < 0.05). *P < 0.05.

METHODS

Subjects. Neonatal male and female inbred C57BL/6J mice were used at postnatal age 9 (n = 132) and 2 days (n = 47) (Jackson Laboratory, Bar Harbor, ME). The EP3R gene was selectively deleted in the EP3R⁻/⁻ mice as described previously (14). Experiments were performed and analyzed after genotype had been determined. All mice were reared by their mothers under standardized conditions with a 12:12-h light-dark cycle. Food and water were provided ad libitum. The studies were performed in accordance with European Community Guidelines and approved by the regional ethic committee. The animals were reared and kept at the Department of Comparative Medicine, Karolinska Institutet.

Reagents. Recombinant mouse interleukin-1β (IL-1β) (Nordic Biosite AB, Täby, Sweden) was reconstituted in sterile pyrogen-free 0.9% NaCl to provide a 1 μg/ml working solution and then stored in −80°C until use. A PGE2 stock solution (20X) was prepared in ethanol and sterile H2O and stored at −20°C. On the day of experimentation, PGE2 was diluted in artificial cerebrospinal fluid (aCSF) to 5 μg/l (9 μM).

Dual-chamber plethysmography in mice. Ventilatory measurements were made using dual-chamber plethysmography in 9-day-old neonatal mice (15). A mask covering the mouth and nostrils was affixed with dental impression material (Impregum F, 3M ESPE). The animals then breathed from one chamber (the headbox) while pressure changes were measured in a second, volume-calibrated plexiglas chamber (35 ml). The signal was digitally converted with a sampling rate of 200 Hz and analyzed using Power Lab software (Chart v5.5.4, PowerLab systems, AD Instruments, Colorado Springs, CO). Ambient temperature within the chamber was measured and recorded continuously using a digital thermometer (Model BAT-12, Physiotemp Instruments) and maintained at 30.1 ± 0.05°C in accordance with the documented thermoneutral range for mice of similar age. The chamber was calibrated repeatedly injecting standardized volumes with preset precision syringes (Hamilton Bonaduz AG).

Plethysmography following intraperitoneal injection of IL-1β or vehicle. Each mouse received an intraperitoneal (ip) injection (0.01 ml/g) of IL-1β (10 μg/kg) or vehicle (NaCl). Baseline skin temperature was recorded immediately prior to injection. After 70-min skin temperature was measured again, and the mouse was fixed within the dual-chamber plethysmograph. Respiration was assessed during 10 min of normoxia (21% O2) followed by 5 min of hypoxia and hypercapnia (10% O2 and 3% CO2), 7 min of normoxia, and then 5 min of hypercapnia (20% O2 and 5% CO2). A recovery period of 5 min in normoxia was followed by a hyperoxic challenge during 1 min (100% O2) and after a final recovery period of 4 min normoxia the animals were subjected to anoxic exposure (100% N2) during 5 min. A subgroup (n = 33) was subjected to different anoxic periods ranging between 6 and 20 min. Finally, 100% O2 was administered for 8 min and the ability to autoresuscitate was evaluated. Skin temperature was recorded at the end of each experiment.

Plethysmography after intracerebroventricular injection of PGE2 or vehicle. After the administration of sevoflurane anesthesia for 60 s, neonatal mice were restrained so that the head was transilluminated with a fiber-optic light on a flexible stem to visualize the lateral ventricles. PGE2 (4 nmol in 2–4 μl of aCSF, n = 19) or vehicle (aCSF, n = 19) was slowly injected into the lateral ventricle by using a thin pulled glass pipette attached to polyethylene tubing (25). The mouse was then placed immediately into the plethysmograph chamber. After a 10-min recovery period in normoxia, the mouse was exposed to a hyperoxic challenge during 1 min (data published in Ref. 25) followed by another 4-min normoxia recovery period and finally subjected to an anoxic challenge (100% N2) for 5 min. Skin temperatures were measured at baseline time points through experimentation and after removal from the chamber by using a thermistor temperature probe. After experimentation animals were dissected at the injection site and examined for the presence of any intracerebroventricular bleeding. Six of 38 mice had minimal but visible bleeding and were excluded from analysis.

Plethysmograph data analysis. Periods of calm respiration without movement artifact were selected for analysis based upon visual observations during experimentation. The values obtained from Power Lab software were then used to calculate mean respiratory frequency (Fr; breaths/min), tidal volume (Vv), minute ventilation (Vv), inspiratory time (Tc), or expiratory time (Te). There was a difference in weight between genotypes, WT vs. EP3R⁻/⁻ (P < 0.05). *P < 0.05.

Table 2. Respiratory responses during normoxia and hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Hypoxia</th>
<th>Normoxia</th>
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<th>Hypoxia</th>
<th>Hypoxia</th>
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<tbody>
<tr>
<td></td>
<td>Fr, breaths/min</td>
<td>Tc, ms</td>
<td>Te, ms</td>
<td>Fr, breaths/min</td>
<td>Tc, ms</td>
</tr>
<tr>
<td>WT NaCl (n = 24)</td>
<td>204 ± 6</td>
<td>37 ± 2</td>
<td>50 ± 4</td>
<td>37 ± 2</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>WT IL-1β (n = 25)</td>
<td>205 ± 6</td>
<td>37 ± 2</td>
<td>51 ± 3</td>
<td>35 ± 2</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>EP3R⁻/⁻ NaCl (n = 21)</td>
<td>192 ± 6</td>
<td>38 ± 2</td>
<td>52 ± 3</td>
<td>37 ± 2</td>
<td>50 ± 3**</td>
</tr>
<tr>
<td>EP3R⁻/⁻ IL-1β (n = 19)</td>
<td>205 ± 7</td>
<td>38 ± 2</td>
<td>56 ± 4</td>
<td>37 ± 2</td>
<td>48 ± 3**</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. All animals respond with an increase in Fr during a moderate hypoxic and mild hypercapnic exposure (10% O2 and 3% CO2). Expiratory time (Te), independent of ip treatment, was longer in EP3R⁻/⁻ mice compared with WT mice (P < 0.01). Inspiratory time (Tc) was similar. **P < 0.01.
quantified using the coefficient of variation (CV) (i.e., SD divided by mean of breath-by-breath interval during 60-s periods).

Brain stem-spinal cord en bloc preparation. Experiments were performed on the brain stem-spinal cord preparation of 2-day-old (P2) pups. The isolated brain stem and cervical spinal cord were obtained under isoflurane anesthesia as described previously (38). The isolated preparations were continuously perfused at a rate of 3.5–4.5 ml/min in a 2-ml chamber with a solution containing (in mM) 130 NaCl, 5.4 KCl, 0.8 KH2PO4, 0.8 CaCl2, 1 MgSO4, 26 NaHCO3, and 30 glucose and equilibrated with 95% O2-5% CO2 at 28°C; pH was set at 7.4 (control aCSF).

In vitro protocols, data recording, and analysis. Inspiratory discharges of respiratory motor neurons were monitored by extracellular recording using glass suction electrodes applied to the proximal cut end of C4 and C3 ventral roots of spinal nerves. Respiratory-related motor neuron burst activity was analyzed and calculated as the number of C4 bursts per minute. After the preparation they were superfused with control aCSF for 40 min and C4 activity reached a steady state, the control perfusate being replaced by testing solutions. Every preparation was exposed only to one testing protocol.

There were three experimental protocols. In protocol 1, the intermittent anoxia consisting of three 3-min intervals of anoxia separated by 5 min of normoxia applied to the preparation. The anoxic solution consisted of aCSF equilibrated with 95% N2-5% CO2. The last anoxic episode was followed by a 30-min interval of washing out with control aCSF.

In protocol 2, the anoxic solution was applied for 15 min followed by control aCSF.

In protocol 3, control aCSF was replaced for 20 min by hypercapnic solution equilibrated with 92% O2 and 8% CO2 (pH 7.2), followed by control aCSF (2).

The control values of the inspiratory burst frequency were calculated during application of normoxic aCSF as the mean of the last 5 min before testing anoxic aCSF application.

During the transient excitatory phase of anoxia, the burst frequency was calculated as the mean over 1 min when the maximal effect was observed. To perform this, the middle of minimal interburst interval was calculated as the mean over 1 min when the maximal effect was observed. To perform this, the middle of minimal interburst interval was calculated as the mean of the last 5 min before testing anoxic aCSF application.

The last anoxic solution was equilibrated with 95% N2-5% CO2. The anoxic solution reached the experimental chamber. The end of the excitatory phase were measured from the moment when anoxic solution reached the experimental chamber. The end of the excitatory phase was defined as the last burst with the previous interburst interval less than 75% of the mean values in control. When hypercapnic solution was applied mean inspiratory frequencies during 5–10 min and the last 5 min were calculated.

Axoscope software and Digidata 1200B interface (Axon Instruments, Foster, CA) were used to collect electrophysiological data. Offline analysis was performed employing Clampfit 8.02 (Axon Instruments, Foster, CA), DATAPAC 2K2 (RUN Technologies, Laguna Hills, CA), and Origin 6.0 (Microcal Software, Northampton, MA) software.

Statistics. Statistical analysis of paired comparisons was performed by Student’s t-test. ANOVA compared those parameters with normal distribution and equal variance, two-way ANOVA was performed where IL-1 altered the response to hypercapnia in WT but not EP3R−/− mice (A and B). Both relative changes in respiratory frequency (FR) from normoxia to hypercapnia (A) and total FR during hypercapnia (B) was altered by IL-1β in WT mice, whereas IL-1β did not alter FR during either normoxia or hypercapnia in EP3R−/− mice (A and B). No differences in tidal volume (VT) (C) or minute ventilation (VE) (D) existed between groups during hypercapnia. Data are presented as means ± SE. *P < 0.05.

Table 3. Respiratory responses during hyperoxia

<table>
<thead>
<tr>
<th></th>
<th>Normoxia Fp, breaths/min</th>
<th>Hyperoxia Fp, breaths/min</th>
<th>Normoxia Fv, breaths/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT NaCl (n = 23)</td>
<td>180 ± 8</td>
<td>158 ± 8</td>
<td>178 ± 9</td>
</tr>
<tr>
<td>WT IL-1β (n = 24)</td>
<td>172 ± 7</td>
<td>145 ± 8</td>
<td>170 ± 8</td>
</tr>
<tr>
<td>EP3R−/− NaCl (n = 20)</td>
<td>161 ± 8</td>
<td>148 ± 9</td>
<td>166 ± 9</td>
</tr>
<tr>
<td>EP3R−/− IL-1β (n = 17)</td>
<td>177 ± 8</td>
<td>160 ± 9</td>
<td>181 ± 10</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. All animals respond with a decrease in Fp during a 1-min hyperoxic exposure (100% O2). No differences in Fp, Fv, or Ve were found between group, irrespective of genotype and treatment.
RESULTS

Animal characteristics. Animal weight was similar between groups even though EP3R/−/− mice were heavier (P < 0.05), a phenotype previously described (25, 42) (Table 1). Weight did not correlate with numbers of gasps, gasping duration, or survival. Finally, there was no difference in weight between males and females, and sex did not affect any of the respiratory variables measured or survival.

Effects of IL-1β on respiration during normoxia and hypoxia. During normoxia both genotypes, irrespective of intraperitoneal treatment, had similar FR, V˙E, VT, Ti, and Tc values (Table 1). During moderate hypoxia and mild hypercapnia, i.e., 5 min of 10% O2 and 3% of CO2, all animals responded with a similar rise in FR from baseline in a range of 10–13% (WT NaCl 13%, WT IL-1β 13%, EP3R/−/− NaCl 10%, EP3R/−/− IL-1β 13%), and without significant changes in V˙E, VT, and Ti. However, Tc was lower in WT mice compared with in EP3R/−/− mice (P < 0.01, Table 2).

IL-1β attenuates the respiratory response to hypercapnia via EP3R. The FR response to hypercapnic challenge (5% CO2 in 20% O2) was attenuated in IL-1β-treated WT mice (Fig. 1A) apparent as a reduced increase in respiratory frequency (P < 0.05, Student’s t-test) as well as a lower respiratory frequency during hypoxia compared with vehicle-treated WT mice (P < 0.05, Student’s t-test, Fig. 1B). The EP3R/−/− mice response to hypercapnia was similar to WT mice but not affected by IL-1β treatment.

Effects of IL-1β on respiration during hyperoxia. All mice responded to hyperoxic challenge with a similar reduction in FR between 8 and 16% (WT NaCl 11%, WT IL-1β 16%, EP3R/−/− NaCl 8%, EP3R/−/− IL-1β 10%, Table 3). An increase in Tc was evident in all the mice within a range of 8–21% (WT NaCl 8%, WT IL-1β 21%, EP3R/−/− NaCl 12%, EP3R/−/− IL-1β 16%). No change in VT, V˙E, or Ti was recorded.

Attenuated EP3R expression enhances respiratory efforts during severe hypoxia. All mice exhibited a biphasic response to severe hypoxia with an initial increase in ventilation (i.e., hyperpnea) followed by hypoxic respiratory depression (i.e.,
primary apnea and gasping) (Figs. 2, A and B). A difference between genotypes in gasping effort was noted, both in duration and total number of gasps. EP3R−/− mice gasped more often compared with WT mice (P < 0.01, Fig. 2C) and also had a longer gasping duration than did WT mice (P < 0.01, Fig. 2D). No difference dependent on treatment was seen. The number of gasps and gasping duration was not correlated to survival, although after 5 min of anoxia almost all animals survived (WT 93% vs. EP3R−/− 84%). EP3R−/− gasped throughout the 5 min of anoxic exposure more often than did the WT mice (WT 38% vs. EP3R−/− 68%, P < 0.05, χ² test).

Response to severe hypoxia in icv-treated animals. During intracerebroventricular (icv) treatment all mice respond in a similar manner during severe hypoxia as described above (Fig. 3, A and B). EP3R−/− mice were able to sustain the hyperpnea period longer than the WT mice irrespective of icv PGE2 or NaCl treatment (P < 0.01, Fig. 3C and Table 4). All animals survived the 5-min anoxic period. EP3R−/− mice took a longer time to autoresuscitate compared with WT mice (P < 0.05), and PGE2 affected the ability to autoresuscitate in WT but not in EP3R−/− mice (P < 0.05, Fig. 3D).

Involvement of EP3R in the hypercapnic response. In brain stem spinal cord preparations during control (normoxic) conditions, the basic burst frequency was similar in WT and EP3R−/− mice [6.73 ± 0.23 bursts/min (n = 16) in WT vs. 7.21 ± 0.33 burst/min (n = 15) in EP3R−/−]. Hypercapnic aCSF (saturated with 8% CO₂) increased inspiratory frequency in WT preparations both after 5 min (P < 0.05) and 15 min exposure (P < 0.001, T-K). Notably, hypercapnia does not affect the inspiratory burst frequency in EP3R−/− mice (P < 0.05, Fig. 4). After 15 min of hypercapnic aCSF, the frequency had increased by 22.1 ± 6.3% in WT (n = 7) vs. 5.8 ± 4% (n = 9) in EP3R−/− preparations compared with control values (Fig. 4).

**EP3R attenuates the initial excitatory phase and reduce the posthypoxic neural arrest.** In both transgenic and WT mouse brain stem preparations the anoxic response was characterized by an initial transient increase in burst frequency and a reduction in burst amplitude (Fig. 5). During prolonged 15-min anoxia the burst amplitude was subsequently restored, and the bursting continued throughout anoxic conditions with a de-

Table 4. Anoxic exposure after icv PGE₂ or NaCl

<table>
<thead>
<tr>
<th>Region</th>
<th>PGE₂ NaCl (n = 6)</th>
<th>WT PGE₂ (n = 7)</th>
<th>EP3R−/− NaCl (n = 9)</th>
<th>EP3R−/− PGE₂ (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyperpnea Duration, s</td>
<td>Total No. of Gasps</td>
<td>Anoxic Period, s</td>
<td></td>
</tr>
<tr>
<td>WT NaCl</td>
<td>47 ± 5</td>
<td>15 ± 3</td>
<td>301 ± 2</td>
<td></td>
</tr>
<tr>
<td>WT PGE₂</td>
<td>51 ± 5</td>
<td>16 ± 3</td>
<td>299 ± 2</td>
<td></td>
</tr>
<tr>
<td>EP3R−/− NaCl</td>
<td>68 ± 4**</td>
<td>17 ± 2</td>
<td>300 ± 2</td>
<td></td>
</tr>
<tr>
<td>EP3R−/− PGE₂</td>
<td>63 ± 4**</td>
<td>15 ± 2</td>
<td>302 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. EP3R−/− mice displayed a longer hyperoxic period during anoxic exposure, independent of intracerebroventricular (icv) treatment (P < 0.01). No differences existed between groups for V̇E or VH. All animals recovered after O₂ was administered. **P < 0.01.
increased frequency as reported previously (2). The mean reduced frequency during the last minute before reoxygenation was similar: 3.59 ± 0.32 bursts/min in WT vs. 3.33 ± 0.52 bursts/min in EP3R−/− preparations.

Resumption of oxygenation during intermittent anoxia induced a posthypoxic neural arrest (PHNA) in 4/10 WT preparations, and in all (n = 8) of EP3R−/− preparations. Moreover, EP3R−/− mice had longer PHNA duration throughout the intermittent anoxia than did WT mice (P < 0.05). Similar results were observed during prolonged 15 min anoxia where duration of PHNA was significantly longer in EP3R−/− mice: 123.4 ± 20.8 s (n = 7) vs. 67.5 ± 8.3 s (n = 6) in WT (P < 0.05, Student’s t-test). Despite this difference in PHNA the inspiratory burst frequency after 4 min recovery in normoxic conditions was similar in both strains (99.7–114.4% of control).

There was no difference between groups in peak frequency during the excitatory phase of the anoxic response. The peak FR of the excitatory phase was reached during development of the anoxic response within a similar time interval in both strains: 66.9 ± 2.12 s in WT preparations vs. 67.3 ± 1.2 s in EP3R−/−. Both during prolonged and intermittent anoxia, the frequency increased up to 310-282% in both strains without effect on number of anoxic episodes during intermittent anoxia. However, the in vitro anoxic response in EP3R−/− mice was characterized by longer initial transient increase in burst frequency compared with WT (Figs. 5 and 6). The whole duration of the excitatory phase was longer in EP3R−/− mice preparations: 154.7 ± 5.2 s (n = 8) in EP3R−/− vs. 133.3 ± 8.3 s (n = 6) in WT (P < 0.05, Student’s t-test).

**DISCUSSION**

Here we demonstrate that IL-1β and PGE2, involved in the development of inflammation, modulate the respiratory activity during hypercapnia as well as severe hypoxia through the EP3 receptor (Fig. 7).

**EP3R alters the response to hypercapnia.** The response to hypercapnia (5% CO2) was attenuated following IL-1β treatment (Fig. 1, A and B). This reduced response to CO2 is likely mediated through EP3R since the IL-1β-induced effect on the hypercapnic response was absent in EP3R−/− mice (Fig. 1). Mice lacking EP3R responded to hypercapnia, independent of treatment, although less than NaCl-treated WT mice. Moreover, the central pattern generator for respiration does not respond to hypercapnia in EP3R−/− mice (Fig. 4). That PGE2 regulates the breathing response to hypoxia is in line with previous data (23, 25, 43). That PGE2 regulates the breathing response to hypercapnia via brain stem EP3R is however a new and unexpected finding (Fig. 1, A and B, and

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**Figure 4.** Hypercapnia increases inspiratory output in WT but not EP3R−/− brain stem preparations. Examples of C4 inspiratory output (A) in brain stem preparations during control conditions (top panel), after 5 min (middle panel), and during the final minute in hypercapnic aCSF (bottom panel). Hypercapnia increases inspiratory frequency in WT mice but does not affect the inspiratory frequency in EP3R−/− mice (B) (P < 0.05). Left panel, the frequency response differed between WT and EP3R−/− mice after 5 min (P < 0.05) and in 15 min (P < 0.001, T-K). Right panel, changes in inspiratory frequency after 15 min of hypercarbic solution. The frequency increased 22.1 ± 6.3% in WT (n = 7) vs. 5.8 ± 4% (n = 9) in EP3R−/− mice compared with control. *Significant difference in WT mice compared with control, and between frequency changes in WT and EP3R−/− mice (P < 0.05, Student’s t-test). Data are presented as means ± SE. *P < 0.05. ***P < 0.001.
Neuronal networks in the retrotrapezoid nucleus/parafascial respiratory group (RTN/pFRG) and the pre-Bötzinger complex (pre-BötC) are critically important in the breathing regulation of CO₂ (21, 56). The robustness and flexibility of effective breathing relies on continual interaction between the RTN/pFRG and the pre-BötC (35). In the RTN, mechanism of chemoreception involves direct H⁺/H₁₁001-mediated activation of chemosensitive neurons and indirect modulation of chemosensitive neurons by purinergic signaling (44). Using WT and EP₃R−/− mice, we previously reported that the EP₃R is expressed in the RVLM, including putative pacemaker neurons in the pre-BötC (25). RTN astrocytes are the source of CO₂-evoked ATP release (19). In the hippocampus EP₃R are expressed and mediate a PGE₂-induced release of glutamate from hippocampal astrocytes (52). If EP₃R receptors are expressed also in RTN astrocytes, an alteration of astrocytic modulators could be an additional possible mechanism for the alteration of CO₂ response that EP₃R mice exhibit.

The RTN/pFGR is vital for the CO₂ response in early life (9). It is also postulated as a conditional oscillator for active expiration quiescent under certain conditions and when turned on stimulates active expiration in adult mammals (26, 40). In our study the expiratory time (Tₑ) was shorter in WT mice compared with EP₃R−/− mice during moderate hypoxia and mild hypercapnia, yielding a potential hypoventilation in the WT mice (Table 2). No difference was found in inspiratory time (Tᵢ).

In preterm infants a blunted response to CO₂ was evident, indicating that apnea of prematurity is caused by an
The present study reveals how the EP3R is involved in the hypercapnic response, enabling respiratory depression, and reduces response to CO₂ changes, proven to be a factor contributing to apneas and SIDS.

**EP3R and PGE₂ release in the immediate response to hypoxia.** When the mice were exposed to hypoxia, previously shown to induce PGE₂ release on its own (43), they initially responded similarly with a hyperpneic period. The hyperpnea was followed by apnea and subsequently the arousal response to hypoxia, considered the last defensive stage, followed by autoresuscitation. Recovering from apnea is enabled through gasping efforts (i.e., deep irregular inspiratory movements with a low frequency involving the whole body and a wide open mouth, Figs. 2, A and B). This ability to initiate appropriate responses to hypoxia is considered a vital reflex in newborns (16). EP3R⁻/⁻ mice gasped more with a longer gasping period, independent of IL-1β or vehicle pretreatment (Fig. 2, C and D). EP3R⁻/⁻ mice were also more prone to continue gasping throughout the anoxic period compared with WT mice. Gasp- ing duration is positively correlated to survival, with both SIDS infants as well as BPD infants having a tendency to exhibit short gasping duration (46).

The present study thus further underlines that a rapid release of PGE₂, and hypoxic activation of mPGES-1, is part of the immediate response to severe hypoxia (3, 23, 25, 43). In addition we demonstrate that PGE₂, via the EP3R, decreases the frequency as well as the duration of gasping efforts.

The EP3R⁻/⁻ mice treated with PGE₂ or NaCl icv displayed a longer period of hyperpneic breathing efforts compared with WT mice (Fig. 3, A–C). A longer duration of central respiratory rhythm excitation was also evident in the brain stem spinal cord preparations (Fig. 5). This was followed by a longer period before autoresuscitation occurred compared with WT mice, evident both in vivo and in vitro (Figs. 3D and 6). In addition, PGE₂ prolonged time to autoresuscitation in WT mice but not in EP3R⁻/⁻ mice (Fig. 3D).

These results are similar to responses to anoxia in mice lacking the adenosine-A1 receptor (Ad-A1R) (27). Both the EP3R and the Ad-AIR are Gi-coupled protein receptors that lower cAMP and reduce neuronal activity, enabling rapid functional recovery of breathing after severe hypoxia. Ad-1R are neuroprotective during acute hypoxia, and lacking them leads to a prolonged hyperpneic response to anoxia and decreased functional recovery (27). In the present study the survival data are inconclusive, and almost all animals survive. This contrasts to data from DBA/1lacJ mice in which PGE₂ affects survival in vivo (25). The difference in outcome could be due to short anoxic exposure (5 min) but is likely explained by strain differences. The DBA/1lacJ mice are hypoxic-sensitive, whereas the present study used C57BL/6J mice that are more sensitive to hypercapnia and less hypoxic-sensitive (23, 53).

The respiratory control is also dependent on dorsal respiratory group, pontine or more rostral brain areas, and peripheral chemoreceptor and pulmonary sensory receptor regulation (34, 36). To our knowledge no data are available of EP3R expression in the peripheral chemoreceptors or pulmonary sensory receptors. PGE₂ is released by carotid body type I cells and exert a paracrine inhibitory effect on the hypoxic response mediated by a G₁ protein EP receptor (17). In neonatal rats inflammation (LPS ip) increases interleukin-1 receptor (IL-1R)
expression in carotid body chemosensitive cells and, in adult mice, IL-1β activation of carotid body IL-1R results in increased sinus nerve activity to NTS and subsequent tachypnea (1, 17) (Fig. 7). Thus, in adult experimental models PGE2 may inhibit and IL-1β increase the carotid body activity. However, the influence of the carotid bodies matures during the first couple of days or weeks after birth (4, 45). The respiratory response of the P9 neonatal mice to physiological denervation of the carotid body, during hyperoxia, suggests that its contribution to the present differences in respiratory responses between the groups is small (Table 3). Furthermore in the isolated brain stem spinal cord preparation the afferent input including carotid bodies as well as pons and suprapontine structures have been removed. The responses in the reduced brain stem preparation are in line with the in vivo data indicating that also in vitro the responses are mainly mediated from the brain stem respiratory rhythm and control centers, the pFRG and the Pre-Bötz (Figs. 4–7).

Implications. Here we show how the respiratory center is depressed via inflammation and hypoxia, leading to hypoventilation, and how this response is altered in EP3R−/− mice. An inability to respond properly to hypoxia may cause recurrent hypoxia, leading to cognitive disabilities in premature infants (33). We also demonstrate how the EP3R has a pivotal role in recognizing hypercapnic changes. The disruption of central CO2 chemosensitivity is a common problem in BPD as well as Rett syndrome and is thought to contribute to the high incidence of unexplained death and abnormal brain development in these children.

In the present study we did not quantify the hypoxic isch-emic encephalopathy after the short anoxic exposure the neonatal mice were exposed to. In the present experiments similar survival rates were noted, and further studies to evaluate the potential long-lasting brain pathology of the anoxic period in relation to respiratory effort and EP3R expression is of interest. Nonetheless, the present data suggest that reducing the activity of the EP3R may decrease life-threatening and brain-damaging hypventilation in neonates. Thus the PGE2 pathway may be a valuable target to steady the breathing of sick babies.

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DISCLOSURES
E. Herlenius is employed at the Karolinska University Hospital and the Karolinska Institutet and is a coinventor of a patent application regarding biomarkers and their relation to breathing disorders, WO2009063226.

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