Adenosine A\textsubscript{2A} receptors mediate GABAergic inhibition of respiration in immature rats

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APNEA OF PREMATURITY IS A common problem among low-birth-weight infants (21). Xanthines, nonspecific adenosine-receptor blockers, are often administered as a treatment in these patients to decrease respiratory irregularity and apneic episodes. However, the precise mechanisms and pathways by which the xanthines act to decrease apnea are not well understood. Adenosine has been shown previously, in several fetal and neonatal animal models, to alter respiratory output by inhibiting respiratory frequency (10, 11, 15). It is therefore likely that the xanthines are exerting their therapeutic effects by blocking the inhibitory effects of adenosine-receptor activation on the respiratory rhythm circuit in the brain stem respiratory rhythm-generating center.

Adenosine can bind to four different adenosine-receptor subtypes, A\textsubscript{1}, A\textsubscript{2A}, A\textsubscript{2B}, and A\textsubscript{3} (4, 23, 26), which are expressed throughout the brain stem (25). Recently, our laboratory has shown A\textsubscript{2A}-receptor message expression in areas of the piglet brain stem involved in respiratory control (31, 32), particularly in areas previously shown to express the inhibitory neurotransmitter GABA in rats (7) and piglets (33). In the piglet, our laboratory has also shown that respiratory inhibition can be elicited by activation of A\textsubscript{2A} receptors and that this effect is blocked by the GABA\textsubscript{A}-receptor antagonist bicuculline (31). However, it is not known whether the inhibitory response to adenosine A\textsubscript{2A}-receptor activation is age or species dependent. Hence, in this study, we tested the hypothesis that the inhibition of respiratory output that occurs with the activation of A\textsubscript{2A} receptors is dependent on GABAergic inhibitory pathways that diminish with advancing postnatal development. To address this hypothesis, we performed experiments in rats of varying ages and administered an adenosine A\textsubscript{2A} agonist before and after GABA\textsubscript{A}-receptor blockade to determine the age-related role of GABA in adenosine-induced respiratory inhibition.

METHODS

Animal preparation. We used Sprague-Dawley rats (Zivic Miller, Zelienople, PA) at three ages: 14 days (P14; ~30 g), 21 days (P21; ~50 g), and adult animals (~250 g) for these studies. All procedures were performed in accordance with protocols that were approved by the Case Western Reserve University Animal Care and Use Committee. Rats were anesthetized with urethane (4 g/kg ip). An arterial catheter was inserted into the carotid artery to allow continuous measurement of blood pressure, and then a bilateral vagotomy was performed to avoid the effect of volume changes on respiratory timing due to stretch reflexes and entrainment of respiratory discharge to the pump rate. A tracheal cannula was then inserted, and the animal was mechanically ventilated by a volume ventilator (Harvard Apparatus, Holliston, MA). Animals were ventilated at a volume of 0.1 ml/10 g body wt. A fine stainless steel bipolar hook electrode was inserted into the costal diaphragm to record electromyographic diaphragm activity, as an index of inspiratory output. Ventilator rate settings were adjusted to give one-third of the maximal diaphragmatic electromyographic amplitude observed in response to 7% CO\textsubscript{2}. Animals were ventilated on room air supplemented by 100% O\textsubscript{2} through a T connection at the ventilator inlet. The atlanto-occipital membrane and occipital bone were removed, and the brain stem and fourth ventricle were exposed. The signal band pass for all electromyographic recording was 0.3–1 kHz, and both the raw and the full-wave rectified averaged signals (50-ms window) were recorded.

Physiological protocols. All drugs were obtained from Sigma Chemical (St. Louis, MO) and injected into the fourth ventricle under direct visualization using a 25-μl Hamilton syringe. The specific adenosine A\textsubscript{2A}-receptor agonist 2-p-(2-carboxyethyl)phenethylaminono-5’-N-ethyl(carboxamido)adenosine hydrochloride [CGS-21680 (CGS)] was dissolved in 100% DMSO and injected (5 μl of 10 mM in P14 and P21 animals, 20 μl of 10 mM in adults) to determine the effects of A\textsubscript{2A} receptor activation on respiration. DMSO was chosen as the vehicle because of insolubility of CGS in aqueous solution except at elevated pH, which can affect respiratory drive. To maintain consistency, DMSO was used as the vehicle for all drugs administered in this study.

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this study. The volume of injection for P14 animals was chosen by a
dose-response curve. A dose-response curve was also performed in
adult animals, using 5-, 20-, and 50-μL injection volumes, none of
which produced a significant change in respiratory frequency (5 μL =
103 ± 4.5%, P < 0.5; 20 μL = 91.6 ± 18.4%, P < 0.7; 50 μL =
131.9 ± 29.8%, P < 0.2). The adult dose was then chosen to be 20
μL, which clearly exceeded the ratio of brain weight change between
P14 and adult animals (P14, 1.15 g; adult, 1.75 g), being 2.5 times the
dose administered for younger animals. Vehicle control injections
were administered using identical volumes to the agonist for each
experiment. The nonspecific adenosine-receptor blocker theophylline
(5 μL of 10 mM) was used to confirm that CGS was exerting its effects
via activation of adenosine receptors. Theophylline was injected
intracisternally in seven additional P14 animals and followed 5 min
later by intracisternal CGS administration. To investigate the role of
GABA in the modulation of respiratory output elicited by A2A-
receptor activation, the GABA α-receptor blocker bicuculline methio-
dide (5 μL of 1 mM) was injected in additional eight P14 and P21
animals followed 5 min later by CGS. A recovery period of 5 min was
allowed between injections. We confirmed that the amplitude of the
diaphragmatic electromyograph had returned to the control level at
this time before further injections. Muscimol (5 μL of 1 mM) was
injected intracisternally in five additional adult animals to investigate
the possibility that they did not respond to CGS injection due to a lack
of diffusion of the drug to respiratory centers.

Data collection and statistics. Diaphragmatic electromyogram was
captured using PowerLab hardware and Chart software in real time at
a 2,000-Hz sampling rate. Data were analyzed on a breath-by-breath
basis for frequency, inspiratory time (Ti), and expiratory times (Te),
normalized to control, and expressed as means ± SE. Control was
taken as the five breaths before the injection of vehicle control or drug.
Animals with a Te longer than 5 s in the control period were not
included in the analysis. Prolonged apnea was defined as Te with a
duration of longer than 15 s. All data were compared using one-way
ANOVA and Tukey’s least squares difference post hoc comparison; P
values ≤ 0.05 were considered significant.

RESULTS

Age-dependent effects of adenosine A2A-receptor activation. Intracisternal injection of vehicle had no effect on respiratory
timing at all ages tested (n = 5 for each age group). Injection
of the adenosine A2A-receptor agonist CGS in P14 animals
resulted in a prolonged apnea (longer than 15 s) in six of nine
rats (Fig. 1A). The other three P14 animals without prolonged
apnea displayed decreased frequency and prolonged Te in
response to CGS administration. Similarly, in P21 animals,
prolonged apnea was observed in four of nine rats (Fig. 1B). Of
the five animals that did not express apneic responses, one
displayed a decrease in frequency and prolonged Te, whereas
the remaining four displayed no measurable change in respira-
tory timing in response to CGS administration. No apneas or
significant changes in respiratory timing were observed in any
of the adult animals when CGS was injected (Fig. 1C) (n = 5).
The time from injection to peak effect of CGS was highly
variable in both P14 and P21 animals with no significant
difference between the two ages. This ranged from 1 s to 2 min
46 s in P14 animals and from 1 s to 2 min 15 s in P21 animals.

Fig. 1. Examples of the effects on integrated (f) diaphragm activity elicited by intracisternal injection of 2-p-(2-carboxyethyl)phenethylamino-5'-N-
ethylcarboxamidoadenosine hydrochloride [CGS-21680 (CGS)] at 3 ages. Intracisternal injection of CGS resulted in apnea these 14-day-old (P14; A) and
21-day-old (P21; B) animals. In contrast, CGS did not affect respiration when injected into adult animals (C). Arrows indicate the time of CGS injection. Time
gap in P14 (A) is 30 min.
The duration of apnea in both P14 and P21 animals was also highly variable, ranging from 16 s to 31 min in P14 animals and from 1 min to 10 min in P21 animals.

Data for frequency and TE for all animals studied are presented in Fig. 2. Apnea is represented by a frequency of 0 Hz and a TE of >15 s. At the time of peak CGS effect, frequency was significantly decreased when expressed as percentage of control before CGS injection in P14 (16.2 ± 9.6%; P < 0.0002), and P21 animals (48.2 ± 15.4%; P < 0.004), but not in adults (91.6 ± 18.4% P < 0.7). As already indicated, CGS resulted in apnea or prolonged TE in all P14 animals. The effect of CGS on TE in P21 animals was variable, and CGS had no effect on TE in adult animals (Fig. 2). CGS also had no measurable effect on Ti in animals that did not become apneic at the three ages examined.

We compared the effects of CGS administration on respiratory frequency immediately before the onset of apnea in P14 and P21 animals. In four of six apneic P14 animals, there was an abrupt onset of apnea with no change in frequency, whereas in two animals there was a modest decrease in frequency before apnea. Overall, frequency before apnea was 93 ± 34% (P < 0.7) compared with control before CGS injection. Unlike P14 animals, P21 animals demonstrated a more gradual entry into prolonged apnea. In all four apneic P21 animals, there was a significant slowing of respiration for 15 s preceding apnea as demonstrated by decreased frequency (60.9 ± 14.4%; P < 0.03) compared with control before CGS injection. There was no measurable effect of CGS on Ti immediately before apnea at either P14 or P21. After prolonged apnea, frequency gradually returned to control within 20 s from resumption of breathing in both P14 and P21 animals.

To determine whether the lack of effect of CGS administration in adult animals was truly age dependent or due to the inability of the drug to reach respiratory centers, we also injected five adult animals with the GABA_A-receptor agonist muscimol. Intracisternal injection of 5 µl of 1 mM muscimol resulted in sustained apnea (longer than 1 min) in four of five animals tested. This volume is one-fourth of that injected for CGS studies and used the same vehicle, suggesting that the intracisternally injected CGS was also accessible to respiratory centers in adult animals.

In seven P14 rats, the nonspecific adenosine-receptor blocker theophylline was used to determine whether CGS was exerting its effects through activation of adenosine receptors. When theophylline was administered 5 min before CGS, the prolonged apneas seen with CGS injection alone were abolished (Fig. 3). Additionally, no significant changes in frequency expressed as percent control (99.7 ± 8.1%; P < 0.9) or TE (91.2 ± 5.7%; P < 0.8) were observed in response to CGS after blockade of adenosine receptors with theophylline. This indicates that CGS-induced inhibition of inspiratory drive is mediated through adenosine receptors.

Effects of GABA_A-receptor blockade on CGS-induced respiratory inhibition. To define the role of GABAergic signaling pathways in CGS-induced respiratory changes, we used the GABA_A-receptor blocker bicuculline. In this set of experiments, bicuculline was injected 5 min before CGS in a separate set of P14 (n = 8) and P21 (n = 8) animals. We used a dose of bicuculline that resulted in a transient increase in frequency. These effects were no longer apparent by 5 min after bicuculline injection, when CGS was administered. Bicuculline completely prevented the prolonged apneas observed with CGS alone in all P14 and P21 animals studied (Fig. 4). Additionally, no significant changes in frequency (P14 = 113.3 ± 9.9%; P < 0.7; P21 = 97.1 ± 3.0%; P < 0.9) or TE (P14 = 95.5 ± 5.4%, P < 0.7; P21 = 105.3 ± 5.8%, P < 0.9) were observed on CGS administration (vs. pre-CGS control periods) after GABA_A-receptor blockade by bicuculline. Therefore, these data indicate that CGS exerted its inhibitory effects by enhanc-
This study demonstrates for the first time that activation of adenosine A2A receptors by the specific agonist CGS-21680 inhibits breathing activity in an age-dependent manner. In all P14 animals, CGS caused a decrease in frequency or apnea, whereas at P21 66% of rats exhibited either a decrease in frequency or apnea in response to CGS. Adult animals exhibit no significant change with CGS administration. Additionally, there were pronounced differences between P14 and P21 animals in the respiratory responses to CGS immediately before prolonged apnea. P14 animals exhibited an abrupt entry into prolonged apnea, whereas P21 animals displayed a significant slowing in respiratory frequency before apnea. Furthermore, the inhibitory effects of CGS could be blocked with the GABAergic-receptor blocker bicuculline, indicating that GABAergic neurons play an important role in this pathway.

Adenosine is formed from nucleotides such as ATP as a natural metabolic breakdown product because of neural activity in the brain (22), and it binds to four different adenosine-receptor subtypes, A1, A2A, A2B, and A3 (4, 23, 26). In general, activation of the A1 receptor has been shown to have a mostly inhibitory effect on neuronal activity (25), whereas the A2 receptors predominantly mediate excitatory influences of adenosine on neurons (10–12, 14, 15). However, in a given brain region, the net response of neuronal discharge to adenosine depends on the balance between its inhibitory and excitatory effects within the local neuronal circuit. Recently, our laboratory has shown that the A2A receptor is expressed in areas of the brain related to respiration (31, 32), which have previously been shown to contain GABAergic neurons in rats (7) and piglets (33). Furthermore, in rat pups the A2A receptor is expressed in GABA-containing neurons that project to inspiratory bulbospinal neurons and the rostral ventrolateral region (32), where the main inspiratory rhythm-generating neurons are located (8, 30).

Previous studies by our laboratory and others, (10, 12, 14, 27, 28, 31) have shown that when adenosine or adenosine analogs are administered to fetal or neonatal animals, a decrease in respiratory output is observed, including a decrease in respiratory frequency. In two of these studies (12, 27), these effects were prevented by pretreatment with the nonspecific adenosine-receptor blocker theophylline. Koos et al. (16) demonstrated that blockade of A2A receptors can prevent hypoxic ventilatory roll-off in young lambs. Additionally, they showed that A2A-receptor activation of peripheral chemoreceptors can modulate respiratory output, resulting in an overall increase in respiratory frequency in fetal sheep (14). However, when the peripheral chemoreceptors are removed, they showed that A2A-receptor activation results in decreased breathing rate by acting centrally at the timing and pattern generating nodes within the respiratory neural network. Our present findings are consistent with these previous studies, because we also observed a centrally induced decrease in respiratory frequency elicited by excitation of A2A receptors in young rats that is blocked by theophylline, although we recognize this to be a
nonspecific blocker of adenosine-receptor subtypes. We have identified a prominent role for A2A-receptor activation in respiratory control during early life, yet our data do not exclude a contribution of A1 receptor activation in neonatal apnea (10, 11, 12).

We postulate that activation of A2A receptors, as demonstrated in our study, exerts its effects on respiration by activation of GABAergic pathways. This is supported by our observation that prior treatment with the GABAA-receptor blocker bicuculline prevented the inhibition of respiratory output seen with CGS alone. The data from this study agree with previous work performed in our laboratory, in which bicuculline also blocked the inhibitory effects of intracisternal CGS administration in piglets (31). However, in our piglet studies no age-related effects of CGS administration were investigated, instead the data captured a very restricted stage of development (2–5 days old). Our present data in developing rats demonstrate that the effects of A2A-receptor activation on respiration are not species specific but age dependent. Studies in other brain regions, such as cerebral cortical neurons, show that bicuculline and picrotoxin antagonize the inhibitory effects of CGS on spontaneous neural firing (24). Moreover, CGS has been shown to promote the release of GABA from striatum and globus pallidus via microdialysis studies (23) and striatal micropunch studies (17). Our recent anatomic and histological evidence supports this mechanism of action in the respiratory system. RNA message for the adenosine A2A receptor is expressed in respiratory-related regions, including the Bötzinger complex, caudal raphe, and gigantocellularis nucleus (32), areas that project to phrenic motoneurons innervating the diaphragm (7). These areas also project to the rostral ventrolateral medulla (2) where respiratory rhythm-generating neurons are located (30). However, because of the nonselective route of drug administration in the present study (i.e., intracisternal injection) the precise anatomic location of CGS and bicuculline action cannot be determined, and in fact they may be acting on multiple sites, resulting in the inhibitory effects seen in these studies.

The present study clearly demonstrates that activation of the A2A receptor is effective in inhibiting respiration in P14 and P21 rat pups but not in adult rats. This, again, is consistent with a previous study by Runold et al. (27) demonstrating that the non-receptor-specific adenosine analog $N^\beta$-sulfophenyl-adenosine was more effective in inhibiting respiratory output in young rabbit pups. The effects of A2A-receptor activation have also shown age dependence in other systems, because CGS increased spontaneous glutamate outflow from the striatum in young but not in old rats (3).

Comparison of the effects of CGS on respiratory timing between P14 and adult rats is more clear cut than between P21 and adult animals. Our data suggest that apnea was more common, frequency decreased to a greater degree, and $\text{Te}$ was...
more prolonged at 14 vs. 21 days. This is consistent with a maturational trend for this phenomenon. The time to onset of apnea was highly variable in both the P14 and P21 groups. However, it was not significantly different between P14 and P21 animals, and it may be due to a difference in diffusion rates of the CGS from the fourth ventricle to respiratory-related centers and possibly related to post-surgical accumulation of fibrin on the brain stem.

One explanation for the age-dependent effects of CGS on respiratory output observed in this study could simply be that there was decreased availability of intracisternally injected CGS to respiratory-related centers of the brain stem in mature animals due to longer diffusion distances or changes in the blood brain barrier. To address this issue, we performed a parallel study in which the GABAergic agonist muscimol was injected into adult animals to determine whether a repeatable response could be elicited using this technique. Muscimol injection resulted in apnea lasting 1 min or more in four of five adult animals. Muscimol was injected in smaller volumes (5 μl vs. 20 μl for CGS) and using the same vehicle as in CGS injections. Taken together, these data indicate that the developmental differences we see in this study are indeed due to biological changes within the respiratory control network and are not simply a limitation of the technique used to administer the drugs. The reasons for this developmental effect of A2A-receptor activation in the respiratory system are not clear, but they may involve one or more of several mechanisms, including 1) a developmental change in A2A-receptor expression in respiratory-related areas as seen in the carotid body (9) or 2) a developmental change in A2A-induced GABA release as seen in the striatum (3).

In preterm infants and in the early postnatal period, inhibitory respiratory reflexes are augmented in response to prolonged hypoxia, hypercapnia, and laryngeal stimulation (1, 6, 20), all of which result in prolongation of TE and therefore may contribute to apnea of prematurity, a common problem among low-birth-weight infants (21). Xanthine therapy is often given to contribute to apnea of prematurity, a common problem among such therapy, and they raise the possibility that development of selective adenosine-receptor subtype antagonists can serve as a better form of therapy for such patients.

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GRANTS

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