Differential Expression of Adenosine A₁ and A_{2A} Receptors After Upper Cervical (C2) Spinal Cord Hemisection in Adult Rats

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Abstract

Background: In an animal model of spinal cord injury, a latent respiratory motor pathway can be pharmacologically activated via adenosine receptors to restore respiratory function after cervical (C2) spinal cord hemisection that paralyzes the hemidiaphragm ipsilateral to injury. Although spinal phrenic motoneurons immunopositive for adenosine receptors have been demonstrated (C3–C5), it is unclear if adenosine receptor protein levels are altered after C2 hemisection and theophylline administration.

Objective: To assess the effects of C2 spinal cord hemisection and theophylline administration on the expression of adenosine receptor proteins.

Methods: Adenosine A₁ and A_{2A} receptor protein levels were assessed in adult rats classified as (a) noninjured and theophylline treated, (b) C2 hemisected, (c) C2 hemisected and administered theophylline orally (3× daily) for 3 days only, and (d) C2 hemisected and administered theophylline (3× daily for 3 days) and assessed 12 days after drug administration. Assessment of A₁ protein levels was carried out via immunohistochemistry and A_{2A} protein levels by densitometry.

Results: Adenosine A₁ protein levels decreased significantly (both ipsilateral and contralateral to injury) after C2 hemisection; however, the decrease was attenuated in hemisected and theophylline-treated animals. Attenuation in adenosine A₁ receptor protein levels persisted when theophylline administration was stopped for 12 days prior to assessment. Adenosine A_{2A} protein levels were unchanged by C2 hemisection; however, theophylline reduced the levels within the phrenic motoneurons. Furthermore, the decrease in A_{2A} levels persisted 12 days after theophylline was withdrawn.

Conclusion: Our findings suggest that theophylline mitigates the effects of C2 hemisection by attenuating the C2 hemisection-induced decrease in A₁ protein levels. Furthermore, A_{2A} protein levels are unaltered by C2 hemisection but decrease after continuous or interrupted theophylline administration. The effects on protein levels may underlie the stimulant actions of theophylline.

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INTRODUCTION

Brain stem bulbospinal neurons located within the dorsal and rostral ventral respiratory groups in the medulla send respiratory inputs to the phrenic motor nucleus in the spinal cord (C3–C5). Monosynaptic connections have been demonstrated between the bulbospinal neurons

and phrenic motoneurons (1,2). These connections mediate the respiratory drive to respiratory motoneurons (3,4). Interruption of central respiratory drive (after high cervical spinal cord injury) invariably leads to respiratory distress, paresis of the diaphragm, or even death if mechanical respiratory support is not provided (5).

In a model of spinal cord injury employed in our laboratory, an upper cervical (C2) spinal cord hemisection interrupts the major descending bulbospinal respiratory motor pathway and paralyzes the hemidiaphragm ipsilateral to the hemisection (6,7). However, respiratory function can be restored to the paralyzed hemidia-

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phragm by activation of a previously quiescent respiratory motor pathway (8–10). The axons of the latent pathway are not transected, because they descend into the cord contralateral to the hemisection, cross the midline, and innervate phrenic motoneurons ipsilateral and caudal to the hemisection. We previously demonstrated that in an animal model of spinal cord injury, this latent respiratory motor pathway can be pharmacologically activated by systemic administration of theophylline leading to restoration of respiratory motor function to a hemidiaphragm paralyzed by C2 spinal cord hemisection (11–13).

Restoration of respiratory activity after systemic administration of theophylline in our model is mediated through antagonism of central adenosine A_1 receptor receptors (12). Although central antagonism of adenosine A_1 receptors mediates functional recovery, it has also been demonstrated that although adenosine A_{2A} receptors do not appear to mediate recovery after injury, activation of peripherally located adenosine A_{2A} receptors by specific agonists can enhance and/or subserve the centrally mediated adenosine A_1 receptor effect (14,15). Recently, by using immunohistochemistry and in-situ hybridization, we demonstrated a positive colocalization of adenosine A_1 receptor mRNA and immunoreactivity (protein) within the phrenic motoneurons of the ventral horns of the cervical spinal cord (16). The finding provided evidence that the adenosine A_1 receptors that likely mediate functional recovery in our model are located in the phrenic motoneurons that innervate the diaphragm.

In a recent study, we provided evidence that the effectiveness of theophylline in restoring respiratory function after C2 hemisection is even more significant than heretofore realized. Specifically, we showed that when rats subjected to a left C2 hemisection were administered theophylline for 3 days and then weaned from the drug for 12 days, the drug-induced recovered activity persisted in the virtual absence of detectable serum levels of the drug (17). In the same study, we reported that adenosine A_1 mRNA levels did not change after theophylline administration over variable periods. We concluded that putative changes in adenosine A_1 and or A_{2A} receptors in the mediation of theophylline-induced effects are post-transcriptional (17). Thus, in the current study, we hypothesize that expression (at protein levels) of adenosine A_1 and A_{2A} receptors will be altered after C2 hemisection and also after systemic theophylline administration.

Here using immunocytochemistry and densitometry semiquantitation, we show for the first time (a) changes in expression of adenosine A_1 and A_{2A} receptors in C2-hemisectioned rats, (b) changes in adenosine A_1 and A_{2A} receptors after uninterrupted administration of theophylline, and (c) changes in adenosine A_1 and A_{2A} receptors when theophylline administration is interrupted.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats (250–300 g) were divided into 6 groups ($n = 4$ –6/group): animals that were subjected to a left C2 hemisection (H), animals that in addition to C2 hemisection received theophylline orally ($3\times$ daily) for 3 days (HT), and hemisectioned animals that received theophylline for 3 days but were weaned for 12 days (HTW) before sacrifice. Appropriate control animals were classified as (nonhemisectioned) untreated animals, animals that received theophylline (orally, $3\times$ daily) for 3 days (T), and animals that received theophylline ($3\times$ daily) and were weaned from the drug for 12 days (TW) before sacrifice.

Prior (10 minutes) to anesthesia, animals were injected intramuscularly with atropine sulfate (0.1 mg/kg) to minimize excessive mucus secretions in the respiratory passages and excessive bleeding during spinal cord hemisection. Animals were then injected with xylazine (7 mg/kg) and ketamine (70 mg/kg) and subsequently subjected to a left C2 hemisection as described elsewhere (18). Postsurgical analgesia (0.05 mg/kg, buprenorphine, subcutaneously) was administered. After completion of the C2 hemisection, animals were returned to clean, litter-lined cages and allowed to recover. Sham-operated animals were subjected to a laminectomy prior to exposure of the C2 and a durotomy only without a C2 hemisection. All animals were carefully monitored during the postsurgical period according to the approved animal protocol and guidelines set by the Division of Laboratory Animal Research at Wayne State University. At 24 hours after C2 hemisection, all animals demonstrated remarkable motor capabilities. Weight loss post injury was minimal (12–18 g). Nonhemisectioned animals were administered theophylline in parallel with all H animals.

Oral Theophylline Administration

Theophylline (1,3-dimethylxanthine; Sigma, St. Louis, MO) was dissolved in physiologic saline (0.9%) and administered orally (20 mg/kg, $3\times$ daily) to experimental and control groups of animals. Animals were classified according to the duration of theophylline administration. Theophylline concentration was calculated such that each animal received a drug volume (mL) equivalent to its body weight (kg). Theophylline was administered orally via gavage (18 gauge, Harvard Apparatus, Holliston, MA). Theophylline or vehicle administration was carried out at 9:00 AM, 1:00 PM, and 5:00 PM.

Animal Perfusion

All animals from all groups were deeply anesthetized with chloral hydrate and intracardially perfused, first with 100 mL of physiologic saline and then with 500 mL of 4% ice-cold paraformaldehyde using a perfusion pump.

Tissue Processing

The spinal cords (C2–C5) were removed and treated with 10%, 20%, and 30% sucrose solution for cryoprotection.

Forty-micrometer thick sections were cut with cryostat and collected as free-floating sections in phosphate buffered saline. Every third 40 μ m was collected. Three series were collected; the first 2 were used to visualize adenosine A₁ and A_{2A} receptors, and the third one was used to control the specificity of the antibody specificity. Analysis consisted of 6 randomly selected sections representative of each area of the cord.

Immunocytochemistry

The sections were incubated overnight in adenosine A₁ (A 268, Sigma) or A_{2A} (A 269, Sigma) primary antibodies (both raised in rabbit antiadenosine A₁ or A_{2A} receptor antibody (Sigma, 1:1000) and subsequently incubated in antirabbit biotinylated antibody (1:200) and the ABC reagent (1:100), both from Vector, Burlingame, CA. The signal was visualized with diaminobenzidine as previously described (19,20). Incubation with omission of primary antibodies (controls) resulted in complete lack of specific labeling.

Quantitation

In sections labeled for adenosine A₁, individual neurons were counted in the ventral horn where the phrenic motor neurons are located. For A_{2A}, the most notable changes were in the white matter ventral and lateral to the dorsal horn, where labeled axons belonging to phrenic motoneurons exit the gray matter. Measurements were taken at this location. For semiquantitative assessment of adenosine A_{2A} immunoreactivity, images were captured using a Leica Axiophot light microscope as previously described (19,20). Images (20–30 through the spinal cord of each animal, respectively) were analyzed with the MetaMorph (Fryer, Huntley, IL) image analysis system, and the relative intensity of the signal was determined. An absolute value was obtained by subtracting the background intensity (measured for each individual section) from the intensity registered in the areas of interest. Individual average intensities were pooled for all animals in each experimental group.

Statistical Analysis.

Statistical analysis (ANOVA) was used to determine differences between groups. Significant difference ($P < 0.05$) between groups was established by the use of the Student-Neuman-Keuls test.

RESULTS

Adenosine A₁ Receptors

In the control animals (T, TW), the immunoreactive cells were located in the ventral horn corresponding to the position of phrenic motor neurons described by us in a previous work (16) (Figure 1). The average numbers ($\pm$ SEM) of cells in 3 control groups were similar: 20 ± 2.1 for noninjured or naïve, 20 ± 2.8 for T, and 17 ± 2.5 for TW.

In the experimental animals, the numbers (of immunoreactive cells) on the ipsi- and contralateral sides

Figure 1. (A) In noninjured animals administered theophylline with or without interruption (T, TW), adenosine A₁ receptor immunoreactivity was unaltered compared with naïve noninjured animals. However, animals subjected to a left C2 hemisection (H) demonstrated a significant (* $P < 0.05$) decrease in protein expression (number of labeled cells) compared with noninjured animals (the decrease was apparent ipsilateral as well as contralateral to injury). Although the decrease on the ipsilateral side appeared more pronounced than that on the contralateral side, this was not significant. After a 3-day administration of theophylline (HT), the decrease in protein levels was significantly attenuated (γ $P < 0.05$) on ipsilateral and contralateral sides. When theophylline was withdrawn for 12 days prior to assessment (HTW), the number of A₁ was still significantly lower compared with H (injury alone). (B) In marked contrast to the decrease in adenosine A₁ protein levels after C2 hemisection (H), adenosine A_{2A} protein levels were not altered compared with controls (T, TW) and with naïve, noninjured animals. However, after theophylline administration, protein levels significantly decreased ($P < 0.05$) ipsilateral as well as contralateral to injury. Although the decrease appeared more pronounced ipsilaterally (a trend similar to that observed for A₁ as shown above), it was not significant. Withdrawal of theophylline for 12 days prior to assessment did not alter the decrease in protein levels, ie, theophylline-induced decrease in A_{2A} protein levels persisted. * = significant from control, γ = significant from hemisection.

Figure 2. Photomicrographs through the ventral roots of the spinal cord caudal to the injury site depicting adenosine A_1 immunopositive neurons (arrows). Note that the number of labeled cells is similar in A (from a

for H rats were 3.5 ± 0.8 and 7.0 ± 1.0 , respectively. This represented a substantially significant reduction by approximately 80% (Figure 1) from naïve animals. Treatment with theophylline (HT) reversed that trend, and the average number of labeled cells was 24.0 ± 1.3 ipsilaterally and 13.0 ± 1.8 in the contralateral gray matter. The HTW animals did not differ noticeably from the HT group: we counted an average of 20.0 ± 0.7 cells ipsilaterally and 17 ± 0.7 contralaterally (Figure 2), and the change in numbers after theophylline administration persisted even when the animals had been weaned from the drug.

Adenosine A_{2A} Receptors

In the white matter (see Materials and Methods), the labeled profiles appear as cross- or longitudinally sectioned axons (21) (Figure 3). The longitudinally sectioned profiles exited the gray matter at its lateral and ventral aspects. The densitometric values (intensity of diaminobenzidine labeling) for the 3 control groups were very similar: 66.5 ± 2.1 for naïve, 70 ± 1.0 for T, and 68 ± 0.08 for TW.

Theophylline administration resulted in a decrease in the intensity of the immunocytochemical reaction, that is, in the HT group measurements were 60.0 ± 2.8 in the ipsilateral and 51.0 ± 1.6 in the contralateral site. Weaning from the drug (TW) resulted in similar values, that is, 62.0 ± 1.5 and 55.0 ± 0.8 for the ipsi- and contralateral sides, respectively.

Hemisection induced a decrease in the intensity of the immunocytochemical reaction by approximately 30% on the ipsilateral side and 15 to 20% on the contralateral side in both groups that received theophylline (Figure 1B).

DISCUSSION

The results from the present study show that basal levels of adenosine A_1 and A_{2A} receptor protein in adult female rats are differentially altered 24 hours after a left C2 hemisection that paralyzes the ipsilateral hemidiaphragm. Specifically, the A_1 but not the A_{2A} receptor protein level is significantly decreased. Furthermore, the change in expression of A_1 after hemisection is reversed after administration of theophylline for 3 days. The changes induced in A_1 expression after theophylline administration persists, despite the fact that weaning from the drug did not further significantly alter A_1 expression. In marked contrast to its actions on A_1

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control animal that has received only theophylline) and C (from an animal that has been hemisectioned and received theophylline). In contrast, in B the number of labeled neurons is significantly reduced (from an animal that was only hemisectioned) but did not receive theophylline. Bar = 50 μ m.

expression, theophylline induced a significant decrease in A_{2A} expression, a decrease that was sustained even after drug administration was withdrawn. Differential expression of A_1 and A_{2A} receptor proteins after hemisection, theophylline administration, and weaning is discussed in terms of adenosinergic mechanisms that may underlie recovery of respiratory function.

Recovery of respiratory-related activity after a left C2 hemisection by a hypoxia-induced activation of a latent respiratory motor pathway has been shown in previous studies (10). Restoration of function after C2 hemisection can also be pharmacologically induced with methylxanthine, theophylline (adenosine A_1 and A_{2A} receptor antagonist), and serotonin (11–13,23,24). Functional recovery is mediated primarily through the blockade of the A_1 receptor subtype, although a permissive/subservient role for adenosine A_{2A} receptors is implicated (12,14). Although involvement of adenosine A_1 receptors in functional recovery after C2 hemisection has been shown, it remains unclear whether recovery was mediated through mRNA levels of adenosine A_1 receptor. It appears that it is unrelated to changes in mRNA synthesis and instead is due to cytoplasmic redistribution of the A_1 protein.

We previously showed for the first time in our model of spinal cord injury, a colocalization of adenosine A_1 receptor mRNA and A_1 immunoreactivity within phrenic motoneurons in the cervical ventral horns (16). In a subsequent series of experiments, adenosine A_1 receptor mRNA levels were assessed at various postinjury intervals to reveal the roles of A_1 receptor mRNA in functional recovery after C2 hemisection. A_1 receptor mRNA levels were not altered by C2 hemisection or theophylline administration at various postinjury times (17). Because adenosine mRNA levels were unchanged after hemisection at various postinjury times and after theophylline administration, we (in agreement with other investigators) determined that the mechanism of theophylline action via the adenosine A_1 receptor subtype could be post-translational (17,25,26). Our findings extend and support previous findings from our lab that postsynaptic mechanisms mediate the effects of theophylline (16). Furthermore, a post-translational involvement was in contrast to other studies that suggested a presynaptic involvement of adenosine A_1 receptors (27).

In this study, the differential effect(s) of hemisection on adenosine A_1 and A_{2A} protein expression and the specific actions of theophylline on the changes extend our previous findings that the A_1 receptors are more implicated than A_{2A} receptors in functional recovery. Reversal of A_1 receptor expression and subsequent persistence of the reversal after theophylline administration strongly imply that (a) the adenosine A_1 protein expression is significantly decreased by injury, (b) reversal of the decrease in expression by theophylline implicates the adenosine A_1 receptor expression post-translationally in functional recovery, and (c) the persistence of A_1

Figure 3. Photomicrographs through the spinal cord of a noninjured animal (A) and caudal to the injury site of an animal that has been hemisectioned and received theophylline (B–D) depicting A_{2A} immunoreactivity. The asterisk in B indicates the label for the contralateral to the hemisection site. Note that the intensity of the reaction is reduced on the ipsilateral side (left) in C compared with D. Arrows indicate longitudinally sectioned axons, and arrowheads point at cross-sectioned axons. The latter are surrounded by nonstained myelin sheaths Bar (insert in D) = 150 μ m in A for A and B, 25 μ m in D for both C and D, and 5 μ m for the inset in D.

protein levels after drug withdrawal indicates an apparent long-lasting effect mediated at the protein level. Moreover, adenosine A_{2A} protein levels remained at or near basal levels 24 hours after injury but decreased after

theophylline administration, a change that was maintained even after drug weaning. Thus, in our model, theophylline exerts unique effects on adenosine A₁ and A_{2A} receptor protein expression after injury. We propose that the differential actions of the drug (shown at the protein level) after injury may be responsible for the functional effects of the drug in restoring respiratory activity after C2 hemisection.

The effects of C2 hemisection on the expression of adenosine receptor proteins reported in the present study contrast with reports in other models of injury. Lai et al (28) showed that after transient ischemic injury, levels of adenosine A₁ protein were markedly raised in the cerebral cortex, whereas Shen et al (29) reported that after ischemia A₁ protein levels were not altered. It is likely that the changes in protein levels post injury depend on the type of injury model and the methodology employed. However, in an ischemic model, Turner et al (30) demonstrated that whereas adenosine A₁ protein levels were decreased by injury, administration of caffeine, a methylxanthine-like theophylline, reversed the decrease, a finding remarkably similar to ours in the present study.

Significance

The findings reported in the present study demonstrate that there is an apparent downregulation of A₁ receptors after C2 hemisection. Downregulation of the A₁ receptors may dampen the known inhibitory actions of endogenous adenosine and could contribute to the hyperexcitability of motoneurons, which causes spasticity after spinal cord injury.

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