Aggravated Brain Damage After Hypoxic Ischemia in Immature Adenosine A2A Knockout Mice

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Background and Purpose—Cerebral hypoxic ischemia (HI) is an important cause of brain injury in the newborn infant. Adenosine is believed to protect against HI brain damage. However, the roles of the different adenosine receptors are unclear, particularly in young animals. We examined the role of adenosine A2A receptors (A2AR) using 7-day-old A2A knockout (A2AR<sup>−/−</sup>) mice in a model of HI.

Methods—HI was induced in 7-day-old CD1 mice by exposure to 8% oxygen for 30 minutes after occlusion of the left common carotid artery. The resulting unilateral focal lesion was evaluated with the use of histopathological scoring and measurements of residual brain areas at 5 days, 3 weeks, and 3 months after HI. Behavioral evaluation of brain injury by locomotor activity, rotarod, and beam-walking test was made 3 weeks and 3 months after HI. Cortical cerebral blood flow, assessed by laser-Doppler flowmetry, and rectal temperature were measured during HI.

Results—Reduction in cortical cerebral blood flow during HI and rectal temperature did not differ between wild-type (A2AR<sup>+/+</sup>) and knockout mice. In the A2AR<sup>−/−</sup> animals, brain injury was aggravated compared with wild-type mice. The A2AR<sup>+/−</sup> mice subjected to HI displayed increased forward locomotion and impaired rotarod performance in adulthood compared with A2AR<sup>++/+</sup> mice subjected to HI, whereas beam-walking performance was similarly defective in both groups.

Conclusions—These results suggest that, in contrast to the situation in adult animals, A2AR play an important protective role in neonatal HI brain injury. (Stroke. 2003;34:739-744.)

Key Words: behavior ■ cerebral ischemia ■ hypoxia ■ newborn ■ mice

Cerebral ischemia is thought of as a disease of the elderly but also commonly occurs in children and often leads to neurological sequelae later in life. Previous studies show that there are important differences between animals of different ages.1-5 It is therefore important to study mechanisms in models of ischemia in the immature brain.

Adenosine is an endogenous neuroprotective agent.6 Adenosine activates receptors of the 4 subtypes A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>, each having a unique distribution in the brain.7 Whereas the neuroprotective role of adenosine is well established in the mature brain, adenosine decreases brain injury via A<sub>1</sub> receptor (A1R) activation, as A1R agonists attenuate and A1R antagonists aggravate brain damage.6 However, in the immature brain, A1R ligands do not affect ischemic brain damage.3,8 The literature on the role of adenosine A2A receptors (A2AR) in cerebral ischemia is less clear-cut. Both A2AR agonists9 and antagonists8,10-13 have shown neuroprotection in models of global brain ischemia. Attenuated focal ischemic brain damage was reported in adult A<sub>3</sub> knockout (A2AR<sup>−/−</sup>) mice compared with wild-type (A2AR<sup>+/+</sup>) mice.14

Given the differences between mature and immature brains and conflicting data on the role of A2AR in cerebral ischemic injuries, we examined the role of this receptor using a previously described model of combined unilateral common carotid ligation and hypoxia1 in 7-day-old A2AR<sup>−/−</sup> and A2AR<sup>+/−</sup> mice.

Materials and Methods

Cerebral Hypoxic Ischemia

We have used a modification1 of a well-established model of focal cerebral hypoxic ischemia (HI) in the 7-day-old rat.15 Mice weighing 4 to 5 g were anesthetized with 1% isoflurane in air. The left common carotid artery was exposed and occluded by electric coagulation, and total surgery time usually did not exceed 2 minutes. Body temperature was maintained at 35°C. Animals that had any bleeding during the surgery were excluded from the study. After surgery, the pups were returned to their dams for 1 hour to recover

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739
from surgery and to feed. They were then placed in an incubator within a chamber, through which a humidified atmosphere of 8% O2 and 92% N2 was transfused. The chamber temperature was kept at 35°C. The pups remained in the hypoxic chamber for 30 minutes. At the end of hypoxia, pups were kept in the incubator in air with temperature maintained at 35°C for 30 minutes (Figure 1B). Experimental protocols were approved by the regional animal ethics committee and followed the European Community regulations.

**Mice**

A2AR(+/+) mice and their A2AR(+/−) controls were backcrossed onto a CD1 (Charles River, Saint Aubin les Elbeuf, France) background for 9 generations.14 Mice were the product of matings between either homozygous A2AR(+/+) or homozygous A2AR(+/−) partners. This was done in no more than 2 generations once separated from the heterozygous line. There was no significant difference in body weight between genotypes at P7 (4.9±0.12 g in A2AR(+/+) and 5.1±0.07 g in A2AR(+/−)).

**Cortical Cerebral Blood Flow Measurement**

In a separate group of mice that was not included in the damage evaluation, we measured bilateral cortical cerebral blood flow (CBF) by laser-Doppler flowmetry (PF3, Perimed) during HI as described.4 Continuous anesthesia (isoflurane 2% for induction and 1% for maintenance) was required to obtain reliable measurements. After 5 days, macroscopic or histopathological brain damage was verified. Since the anesthesia during hypoxia might have influenced the severity of brain damage in these mice, they were not included in the main evaluation of brain injury.

**Temperature**

Rectal temperature was examined in a subgroup of the animals included in the brain injury evaluation. After electrocauterization of the left common carotid artery as described, rectal temperature was measured during exposure to 30-minute hypoxia and 2.5 hours thereafter with a BAT-10 (Physitemp) thermometer with a superfine probe (RET-3).

**Brain Damage Evaluation**

Animals were killed by intravenous injection of pentobarbital 5 days, 3 weeks, or 3 months after HI and were perfused through the left ventricle with 4% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). At 5 days after HI, brain damage was evaluated by means of a gross morphology score described for HI in the rat brain.8 Brains were given the score 0 if the 2 hemispheres were equal in size, 1 for hypotrophy of the lateral posterior part of the left hemisphere, 2 for atrophy of the anterior and posterior parts of the left hemisphere, or 3 for large cysts in the left hemisphere. The brains were kept in fixative for approximately 3 hours at 4°C, then transferred into 30% sucrose until frozen on dry ice and cut in 20-μm coronal sections. In a subgroup of these brains, a histopathology score and regional area measurements were taken on cresyl violet–stained sections. In another set of animals, brain damage was evaluated after 3 weeks or 3 months by means of histopathological scoring and area measurements.

The histopathological damage was scored with the use of light microscopy on a 0- to 24-point scale total for each brain. To derive the total score, 8 brain regions most affected by HI were scored on a scale of 0 to 3 and summed.11 Scoring was made by an investigator unaware of the animal group assignment. A computerized image analysis system (Imaging Research Systems) was used to measure the area of intact tissue in cortex and striatum and the residual tissue in hippocampus at 3 different coronal levels, shown in Figure 2.

**Behavioral Tests**

Behavioral tests were performed after HI in 2 different sets of animals, at age 4 weeks and at age 3 months, and were evaluated by an investigator unaware of the animal group assignment. Controls
were not subjected to HI. Locomotor activity and beam walking were performed as previously described. In the locomotor activity experiments, the dopamine D, agonist SKF 82958 C (6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetra-hydro-1H-3-benzazepine hydrobromide; RBI; 1 mg/kg IP) was used as a motor stimulant on the basis of a report by Chen et al showing that D, agonists have similar effects on behavior in adult A2AR(+) and A2AR(−/−) mice.

Three days after the locomotor activity test and beam-walking test, animals were tested on an accelerating rotarod (Panlab s.l.). Animals were habituated on the rotating drum at a constant speed (4 rpm) for at least 30 seconds, and then the rotarod accelerated from 4 to 40 rpm over 5 minutes. The time until the mouse fell off the drum was recorded in 5 consecutive sessions, and the highest value for each animal was used for statistical calculations.

Statistical Analysis

All values are expressed as mean±SEM. Statistical procedures in the software packages Systat (Systat, Inc) and Graph Pad Prism (Graph Pad Software, Inc) were used. Differences in mortality between genotypes was analyzed by x² test. Gross morphology score and histopathology score were analyzed by Mann-Whitney U test. Regional area measurements were compared between A2AR(+/+) and A2AR(−/−) mice by 2-tailed t tests. Data from behavioral tests were analyzed by 1-way ANOVA with Tukey’s post hoc test. Data from CBF and temperature measurements were analyzed by repeated-measures ANOVA. A value of *P<0.05 was considered significant.

Results

Cortical CBF, Rectal Temperature, and Mortality During Hypoxia

A2AR are present on blood vessels and also mediate vasodilation in the central nervous system. Therefore we investigated the effect of 30 minutes of HI on cortical CBF in anesthetized animals. As shown in Figure 1A, cortical CBF was reduced to a similar extent in A2AR(−/−) and A2AR(+/+) mice with 5 minutes of hypoxia and was similarly normalized in both groups within 5 minutes after the animals were returned into normoxic conditions. Rectal temperature was studied during and for 2 hours after hypoxia (Figure 1B) and did not differ between genotypes.

During the period of hypoxia, 13 of the 71 A2AR(+/+) animals and 5 of the 66 A2AR(−/−) mice died. Thus, intrahypoxic mortality was slightly higher in the A2AR(−/−) group (18%) than in the A2AR(+/+) group (8%; *P<0.001). In the 5-day period after HI, an additional 6 of the A2AR(−/−) and 5 of the A2AR(+/+) animals died.

Brain Damage Evaluation 5 Days After HI

In a total of 54 mice, brain damage was evaluated 5 days after lesion by means of gross morphology score, histopathology score, and

| TABLE 1. Extent of Damage in A2AR(+/+) and A2AR(−/−) by Various Scores |
|---------------------------------|-----------------|-----------------|
| Score/Time                      | A2AR(+/+) Mean | A2AR(−/−) Mean |
|                                 | (median, range) | (median, range) |
| Gross morphology score (0–3)   | 1.1 (1, 0–3, n=32) | 2.0** (2, 0–3, n=22) |
| 5 days after HI                 |                 |                 |
| Histopathology score (0–24)    | 5.5 (1, 0–23, n=20) | 15.1* (20, 0–23, n=9) |
| 5 days after HI                 |                 |                 |
| Histopathology score (0–24)    | 9.7 (7, 0–24, n=19) | 19.5* (19.5, 2–24, n=10) |
| 3 weeks after HI               | 10.7 (11, 0–21, n=7) | 18.9 (20, 10–24, n=7) |

A significant difference between genotypes in gross morphology score and histopathology score was found at 5 days and 3 weeks after hypoxic ischemia (HI).

*P<0.05, **P<0.01.

| TABLE 2. Damage as Assessed by Cross-Sectional Area |
|---------------------------------|-----------------|-----------------|
|                                | A2AR(+/+) Ipsilateral, mm² | A2AR(−/−) Ipsilateral, mm² | A2AR(+/+) % Ipsilateral Damage (95% CI) | A2AR(−/−) % Ipsilateral Damage (95% CI) |
| 5 days after HI                 | n=18            | n=6–7           |                                |                                 |
| Cortex                         | 5.1±0.4         | 2.8±0.6**       | 11.9 (–3.1–26.8)               | 44.2 (11.3–77.1)                |
| Striatum                       | 4.2±0.3         | 3.0±0.4*        | 9.4 (–2.3–21.2)                | 40.9 (21.0–60.7)                |
| Hippocampus                    | 0.7±0.1         | 0.2±0.1*        | 31.7 (15.1–48.4)               | 71.4 (43.4–99.4)                |
| 3 weeks after HI               | n=17            | n=11            |                                |                                 |
| Cortex                         | 7.9±0.6         | 5.8±1.2         | 16.2 (3.7–28.8)                | 37.3 (11.0–63.5)                |
| Striatum                       | 5.2±0.3         | 4.6±0.5         | 20.5 (10.2–30.8)               | 18.1 (–1.4–37.5)                |
| Hippocampus                    | 0.7±0.1         | 0.3±0.1*        | 48.5 (31.8–65.2)               | 71.3 (50.8–91.8)                |
| 3 months after HI              | n=7             | n=7             |                                |                                 |
| Cortex                         | 7.9±1.2         | 5.2±1.2         | 9.8 (–18.1–37.6)               | 44.0 (5.9–82.0)                 |
| Striatum                       | 5.7±0.8         | 4.1±0.9         | 6.7 (–15.1–28.4)               | 24.5 (–16.7–65.7)               |
| Hippocampus                    | 2.1±0.9         | 0.6±0.2         | 38.7 (–2.6–79.9)               | 71.2 (40.2–102.2)               |

Percent ipsilateral damage was calculated using the formula: % damage=100×(right-sided area–left-sided area)/right-sided area. Areas of nonaffected regions from the middle coronal section (see Figure 2) are shown, but the same results were found in data from all sections.

HI indicates hypoxic ischemia.

*P<0.05, **P<0.01.
regional area measurements. In 2 A2AR−/− and 28 A2AR+/+ mice, there was no evident macroscopic brain damage.

The mean gross morphology score was significantly higher (indicating more severe damage) in A2AR−/− than in A2AR+/+ CD1 mice (Table 1). Microscopic brain damage was detected in all A2AR−/− animals except 1 and in 8 of 20 A2AR+/+ animals. Because of the 18% mortality during hypoxia among A2AR−/− mice, we were reluctant to prolong the duration of hypoxia to achieve a more clear-cut effect in the A2AR+/+ animals. Histopathological score was significantly higher (indicating more severe damage) in A2AR−/− than in A2AR+/+ mice (Table 1). Most A2AR−/− mice displayed severe damage with a cystic infarction affecting ipsilateral hippocampus, cortex, and striatum, whereas A2AR+/+ mice displayed a milder injury confined to hippocampus. There was no evident brain damage on the contralateral side.

There was a positive correlation between gross morphology score and histopathology score (r=0.78, P<0.001, Spearman correlation), indicating that macroscopic injury is a good indicator of histopathological damage in the present model.

Comparison of regional cross-sectional area confirmed exacerbated damage in the A2AR−/− group 5 days after lesion. The residual cross-sectional areas of ipsilateral cortex, striatum, and hippocampus at the 3 levels indicated in Figure 2 were all significantly reduced in A2AR−/− mice compared with A2AR+/+ mice (Table 2).

Brain Damage Evaluation 3 Weeks After HI
To examine whether the aggravation of the brain injury in A2AR−/− mice persisted, we evaluated the histopathological brain damage 3 weeks and 3 months after HI in animals that had undergone behavioral tests. Histopathological scoring 3 weeks after HI revealed a pattern of damage similar to that after 5 days: A2AR−/− mice displayed significantly more damage than A2AR+/+ mice (Table 1). Additionally, regional cross-sectional area measurements confirmed more severe injury in A2AR−/− mice in the hippocampus 3 weeks after HI (Table 2).

Brain Damage Evaluation 3 Months After HI
Even when studied 3 months after lesion, there was a trend toward increased severity of brain damage in A2AR−/− mice compared with A2AR+/+ mice (Table 1), but this was not significant (P=0.082, Mann-Whitney U test), probably because of the limited number of animals studied at this time point. The same (nonsignificant) trend was seen in the regional area measurements (Table 2).

Behavioral Evaluation
Forward Locomotion
Forward locomotion was measured during 3 periods of habituation to the test box and once after the administration of the D1 agonist SKF 82958 C.2 Even in control animals not subjected to HI, forward locomotion was slightly, but significantly (P<0.05), increased in 4-week-old A2AR−/− controls compared with A2AR+/+ controls (Figure 3A). This tendency was seen during all the test periods, including the period after SKF 82958 C (not shown). In agreement with a previous report,18 we found that this drug has a similar stimulating effect on locomotor activity in A2AR−/− and A2AR+/+ mice.

Among animals exposed to HI 3 weeks earlier, forward locomotion was increased in the A2AR−/− group, whereas A2AR+/+ mice exposed to HI had a tendency to decreased forward locomotion (Figure 3A). Forward locomotion was significantly increased in A2AR−/− mice that had undergone HI compared with A2AR+/+ mice (Figure 3A). The A2AR−/− mice, especially after HI, habituated to the environment more slowly than A2AR+/+ mice (not shown).
In another set of mice that were 3 months old, the same tendency toward an increased forward locomotion was seen in the A2AR$^{-/-}$ controls (not subjected to HI) compared with A2AR$^{+/+}$ controls during the second habituation (Figure 3A). Among animals that were subjected to HI 3 months earlier, forward locomotion was further increased, as shown by a significant ($P<0.005$) interaction between treatment and genotype in the ANOVA test (Figure 3A).

**Rotarod Performance**

Untreated 4-week-old A2AR$^{-/-}$ mice had a nonsignificant tendency to stay on the accelerating rotarod longer than A2AR$^{+/+}$, but 3 weeks after HI, A2AR$^{-/-}$ mice had a tendency to perform worse than controls of the same genotype (Figure 3B). This impairment was significant 3 months after HI (Figure 3B). A2AR$^{+/+}$ mice that had been subjected to HI were not affected in the rotarod test at any time point studied.

**Beam-Walking Performance**

The total number of slips (as well as slips on the affected side; not shown) was higher in animals that had been subjected to HI, both after 3 weeks and after 3 months (Figure 3C; $P<0.001$). However, there was no difference between genotypes. Data from habituation 2 are shown, but the effect of HI on number of total and right-sided slips was similarly significant for all habituations.

**Discussion**

Cerebral HI is an important cause of brain injury in the newborn infant. In the present study we hypothesized that A2AR play a crucial pathophysiological role in HI of the immature brain. The major finding was that brain injury after neonatal HI was aggravated in A2AR$^{-/-}$ compared with A2AR$^{+/+}$ mice. In particular, we showed that the brain damage persisted at least up to 3 weeks after HI and that it was related to behavioral alteration over the short and long term.

More severe damage in mice with a targeted disruption of the A2AR gene is in apparent contrast to studies of focal brain ischemia in adult A2AR$^{-/-}$, in which A2AR inactivation resulted in reduced brain damage and improved neurological score evaluated 5 days after ischemia.14 The possibilities that the divergent results may be due to age or strain differences are discussed in relation to the actions of A2AR.

It is well known from previous studies that the mechanisms underlying posts ischemic brain damage1,5,19 as well as the role of adenosine receptors5 differ in many ways between young and old mice (see below). An age-dependent difference in A2AR is also indicated by the fact that in adult rats an A2AR antagonist has a clear cerebroprotective effect,13 consistent with the findings in adult A2AR$^{-/-}$ mice,14 whereas pretreatment of immature animals with the same antagonist has no protective effect.8

It is also conceivable that a targeted disruption of the A2AR could yield different phenotypes in different background strains.26 In the present study we used A2AR$^{-/-}$ mice on a pure CD1 background, whereas in the report by Chen et al.14 C57BL/6X129 Steel and pure 129 Steel mice were examined. The previous data in both immature17 and adult mice21 show that CD1 and C57BL6 strains are similarly sensitive to ischemia. However, the 129 strain is substantially more resistant to ischemic brain damage.17,21 Nevertheless, the effect of ischemic brain injury in adult mice was equal in A2AR$^{-/-}$ from C57BL/6X129 Steel and pure 129 Steel mice.14 This argues strongly that the effect of A2AR deletion is more important than the genetic background. The fact that there are similarities in the effects of A2AR antagonists in adult rats13 and of A2AR disruption14 in adult mice also suggests that the role of A2AR transcends species barriers, thereby making strain differences less likely. Nevertheless, it may be important in future experiments to examine how adenosine receptors interact with other genes, and a systematic examination of the magnitude of A2AR effects in mice with different genetic backgrounds might give interesting results.

In addition to age-dependent differences in the role of A2AR, there is evidence for an age-dependent difference in the role of A1R. Whereas stimulation of A1R protects the adult brain from ischemic injury,6 A1R play a minor role in the immature brain.5,8 Thus, although there is a report suggesting small adaptive changes in A1R in adult A2AR$^{-/-}$ animals,22 such changes are not likely to affect brain damage in the brains of immature A2AR$^{-/-}$ animals. Instead, the lack of functional A1R may be speculated to leave room for an increased relative importance and a protective role of A2AR in the immature brain.

The morphological damage was most prominent in the hippocampus and in the cortex, but A2AR are predominantly located in the striatum. The lack of correlation between distribution of morphological damage and neuronal A2AR suggests that the mechanism for an aggravated injury in A2AR$^{-/-}$ mice is nonneuronal. Indeed, A2AR stimulation mediates vasodilation and inhibition of neutrophil activation, and both these actions might account for detrimental effects of A2AR deficiency in focal brain ischemia. In the present study direct comparison of cortical CBF during hypoxia showed no difference between surviving A2AR$^{-/-}$ and A2AR$^{+/+}$ animals. However, we cannot rule out the possibility of an altered CBF in A2AR$^{-/-}$ mice at some stage after hypoxia.

Neutrophils are thought to be involved in the pathophysiology of HI brain injury in mature as well as immature animals and are present 12 hours after reperfusion in the P7 rat brain.19 Release of free radicals from activated neutrophils and reperfusion injury in the canine heart are both attenuated by A2AR activation.23 Furthermore, it is shown that cell death and proinflammatory events after intracerebral hemorrhage were decreased after administration of an A2AR agonist.24 There is actually evidence for a particular susceptibility to free radicals in the immature brain compared with the mature brain, probably due to immaturity of catalytic enzymes.5 Therefore, a lack of A2AR on, for example, neutrophils in the immature brain is a possible explanation for the diverse results obtained in the present immature ischemia model and in adult animals.

**Behavioral Evaluation**

There are few reports on functional deficits after focal ischemia, and even fewer have used neonatal models of ischemia.25,26 The present study is the first to demonstrate clear functional effects of ischemic brain injury at P7 that remain into adult life.

As expected, an impaired sensorimotor function assessed by rotarod and beam-walking test was maintained long after injury. The beam-walking test might actually be more sensitive to...
minor ischemic damage than the rotarod and locomotor activity tests, since animals with both mild and severe brain injury were impaired in this test.

Since a recent study reported decreased locomotor activity 24 hours after focal ischemia with a middle cerebral artery occlusion model in wild-type CD1 mice, the present result of increased locomotor activity in the injured A2AR−/− mice was not expected. The increased locomotion in the A2AR−/− mice might be due to a general increase in motor activity. Thus, we found that there was a small increase in basal locomotor activity/forward locomotion at 4 weeks of age in the A2AR−/− controls compared with A2AR+/− controls. However, increased locomotor activity is not a general characteristic of A2AR−/− mice. It should be emphasized, though, that a major physiological role of A2AR stimulation is to modulate locomotor activity. In particular, it is known that A2AR blockade potentiates locomotor effects of dopaminergic agents. Thus, if endogenous dopaminergic tone is increased, one might expect A2AR−/− mice to exhibit higher motor activity.

A previous study in adult gerbils has shown increased locomotor activity after global brain ischemia. These results and those of the present study may be related to the cortical and hippocampal damage. It has been proposed that the increased locomotion reflects a deficit in habituation or spatial mapping rather than primarily a defect in sensorimotor function. This would theoretically lead to a slower adaptation to the test environment, and, in the present study, the A2AR−/− mice habituated more slowly than A2AR+/− mice at all time points studied.

In conclusion, the present results show that A2AR deficiency aggravates the damage and long-term functional consequences thereof in a mouse model of HI in the neonatal brain. In accord with the established role of adenosine as an endogenous neuroprotective agent, our results suggest that, in the immature brain, A2AR stimulation during HI is beneficial.

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