Amiloride Improves Locomotor Recovery after Spinal Cord Injury

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Abstract

Amiloride is a drug approved by the United States Food and Drug Administration, which has shown neuroprotective effects in different neuropathological conditions, including brain injury or brain ischemia, but has not been tested in spinal cord injury (SCI). We tested amiloride's therapeutic potential in a clinically relevant rat model of contusion SCI inflicted at the thoracic segment T10. Rats receiving daily administration of amiloride from 24 h to 35 days after SCI exhibited a significant improvement in hindlimb locomotor ability at 21, 28, and 35 days after injury, when compared to vehicle-treated SCI rats. Rats receiving amiloride treatment also exhibited a significant increase in myelin oligodendrocyte glycoprotein (MOG) levels 35 days after SCI at the site of injury (T10) when compared to vehicle-treated controls, which indicated a partial reverse in the decrease of MOG observed with injury. Our data indicate that higher levels of MOG correlate with improved locomotor recovery after SCI, and that this may explain the beneficial effects of amiloride after SCI. Given that amiloride treatment after SCI caused a significant preservation of myelin levels, and improved locomotor recovery, it should be considered as a possible therapeutic intervention after SCI.

Key words: amiloride; myelin; SCI

Introduction

AMILORIDE is a potassium-sparing mild diuretic, approved by the United States Food and Drug Administration, that has been used in combination with other diuretics in the treatment of hypertension since 1967 (Arias et al., 2008; Baer et al., 1967; LaGow et al., 2008). Additionally, amiloride can cross the blood–brain barrier and has proven beneficial in the treatment of several neuropathological conditions including brain ischemia (Xiong et al., 2004), multiple sclerosis (Friese et al., 2007; Vergo et al., 2011), seizures (Ali et al., 2004, 2005, 2007; N'Gouemo, 2008), Parkinson's disease (Arias et al., 2008), gliomas (Sipos et al., 2000), and in an *in vitro* model of spinal cord dorsal column compression injury (Agrawal and Fehlings, 1996). However, the therapeutic potential of amiloride has not been tested in a clinically relevant animal model of contusion spinal cord injury (SCI).

Amiloride exerts its neuroprotective effects by collectively inhibiting several pathways, whose simultaneous activation leads to a highly toxic intracellular cation overload; i.e., amiloride inhibits acid-sensing ion channels (ASICs), Na⁺/H⁺ exchangers (NHE), Na⁺/Ca²⁺ exchangers (NCX) (Xiong

et al., 2008), and voltage-gated Na⁺ channels (Kleyman and Cragoe, 1988).

ASICs are proton-gated channels permeable to cations upon activation by decreased extracellular pH (Xiong et al., 2004). Although individual ASIC channels become activated at different extracellular pH values, even a mild reduction in parenchymal pH (<6.9) is sufficient to activate ASIC1a (Waldmann, 2001), whose expression has been shown in spinal cord neurons (Duan et al., 2007). ASIC1a is permeable to Na⁺ and Ca²⁺, whereas other ASIC isoforms are only permeable to Na⁺ (Carnally et al., 2008; Waldmann 2001). It has been shown that blocking acidosis-induced cation entry through ASIC1a reduces intracellular cation overload and thus prevents acidosis-induced toxicity and cell death (Pignataro et al., 2007; Xiong et al., 2004, 2006, 2008; Yermolaieva et al., 2004). ASICs are activated in different neuropathological conditions by acidosis generated from hypoxia or inflammation (Reeh and Steen 1996; Siesjo et al., 1996), both robustly initiated after SCI (Stys 1998; Trivedi et al., 2006). For example, in the acute post-SCI phase, Farooque and associates (1996) found that extracellular lactate levels increase five- to sixfold, which lowers parenchymal pH sufficiently to activate several ASICs. However, extracellular acidosis is likely to

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persist in injured spinal cords because of persistent hypoxic and inflammatory conditions, although direct measurements of the parenchymal pH levels after SCI have not been performed. Despite its likely contribution to SCI-induced cell loss or dysfunction, the role of ASICs in SCI has not been investigated.

Whereas ASICs are activated by extracellular acidity, NHEs are activated by intracellular acidity, which also accompanies hypoxia (Yao and Haddad, 2004). The NHEs are ubiquitously expressed within the central nervous system (CNS) (Luo and Sun, 2007), and normally regulate intracellular pH by exchanging one intracellular H^+ with one extracellular Na^+ (Obara et al., 2008). Decreased intracellular pH and induction of NHE activity can lead to high levels of intracellular Na^+ if adequate levels of ATP are not available for Na^+K^+ ATPase activity (Jung et al., 2007). Given that hypoxic conditions with insufficient levels of ATP have been documented in injured spinal cords (Anderson et al., 1980; Nesic et al., 2008; Xiaowei et al., 2006), it is likely that NHEs are not only chronically activated after SCI, but that their activation results in the sodium overload in all NHE-expressing cells. Despite its clear significance, NHE inhibition in SCI has not been studied.

Combined activation of NHEs and ASICs after SCI will lead to Na^+ overload, which in turn will induce the reverse operation of the NCX, during which the NCX brings Ca^{2+} into the cell as Na^+ is extruded (Annunziato et al., 2004). Therefore, cells in injured spinal cords are not only subjected to the overload of sodium, but also to the even more harmful overload of calcium (Stys et al., 2004). The reverse operation of the NCX after SCI has been shown to contribute to increased intracellular Ca^{2+} levels and toxicity specifically in the white matter (Annunziato et al., 2004; Li et al., 2000), because the NCX is highly expressed in myelinated axons (Steffensen et al., 1997) and oligodendrocytes (Chen et al., 2007; Quednau et al., 1997). Various NCX inhibitors have been shown to decrease white matter damage after hypoxic, ischemic, or compressive injury to isolated spinal cord (Annunziato et al., 2004; Ouardouz et al., 2005; Tomes and Agrawal, 2002;), but have not been investigated in contusion SCI.

Therefore it is not surprising that amiloride, a potent inhibitor of ASICs, NHE, and NCX, has neuroprotective effects in many pathological conditions associated with hypoxia and inflammation, including SCI, as presented here. However, amiloride can also inhibit urokinase-type plasminogen activators (uPA) (Vassalli and Belin, 1987), adenosine A1 receptors (Garritsen et al., 1992), or GABA_A receptors (Fisher, 2002), and therefore it is possible that those targets also contribute to the amiloride-induced neuroprotection; but this remains to be analyzed in further studies.

Amiloride Improves Locomotor Recovery

A 10 mM amiloride (Sigma A7410) solution was made daily by dissolving in water. Daily i.p. injections of amiloride or vehicle ($n=6$ /group) began 24 h after injury, and continued until 35 days after injury when animals were perfused and used for Western blot analysis. Amiloride is not significantly metabolized (LaGow et al., 2008; McGuigan, 1997); i.e., it is eliminated from the body unchanged in urine (50%) and feces (40%). It has a half-life of 6–9 h after administration; therefore, daily injections of free amiloride were necessary. As described

in the introductory paragraphs, amiloride can affect various targets expressed in different cell types, and thus it can modulate pathophysiological processes occurring at different times after SCI. Amiloride can affect neuronal death (Friese et al., 2007); therefore, acute intervention is warranted. But it can also affect functions of surviving axons (Agrawal and Fehlings, 1996), and therefore chronic amiloride intervention is justified. Therefore, we have decided to administer amiloride from 24 h until 35 days after SCI.

Amiloride was administered at a dose of 5 mg/kg for the first 10 days after injury, after which the daily dose was increased to 10 mg/kg for days 11–35 after injury. A dose of 10 mg/kg was previously found to be neuroprotective in a mouse model of multiple sclerosis (Friese et al., 2007), and a mouse model of Parkinson's disease (Arias et al., 2008). Amiloride is an aromatic drug that can diffuse across membranes and accumulate within cells (Kleyman and Cragoe, 1988), allowing it to cross the blood–brain barrier at sufficient doses. Seizure studies in mice suggest that amiloride crosses the blood–brain barrier when administered at 0.65 mg/kg i.v., causing anticonvulsant actions by acting on CNS targets (Ali et al., 2007). Similarly, other studies suggest that amiloride crosses the blood–brain and blood–spinal cord barriers, exerting neuroprotective effects when administered systemically at 10 mg/kg i.p. (Arias et al., 2008; Friese et al., 2007; Vergo et al., 2011). Therefore, the doses of amiloride used in our study (5 and 10 mg/kg i.p.) probably penetrated the blood–spinal cord barrier (BSCB); especially considering that the BSCB has increased permeability at the lesion epicenter up to 7 days after SCI (Cohen et al., 2009; Popovich et al., 1996; Whetstone et al., 2003), and remains compromised even 56 days after SCI (Cohen et al., 2009).

However, our experiments found that 10 mg/kg daily i.p. injections of amiloride could be harmful when the first injection was given immediately or 24 h after SCI. Naïve animals were not harmed by daily amiloride i.p. injections of 10 mg/kg, suggesting that SCI animals in particular are more sensitive to amiloride doses at early time points after injury. Amiloride can lead to hyperkalemia and cardiac complications, especially in patients with renal impairment (LaGow et al., 2008). Because impaired renal function occurs in acute SCI (Ruffion et al., 2007; Samson and Cardenas, 2007; Vaziri et al., 1984), it is not surprising that acutely injured rats were more sensitive to amiloride treatment. Therefore, we first injected a lower dose of amiloride (5 mg/kg), followed by a higher dose of amiloride (10 mg/kg) at 11 days after SCI, when kidney and bladder function had stabilized (see Fig. 1B). This administration paradigm was not only well tolerated by SCI rats, but also produced a significant improvement of motor recovery after SCI (Fig. 1A).

All SCI rats (in both vehicle- and amiloride-treated groups) had indistinguishable Basso, Beattie, and Bresnahan (BBB) scores for the first 2 days after SCI (Fig. 1A), indicating that the initial contusion injury was similar in all rats. Although the BBB scores became higher in the amiloride treated group after day 7, locomotor recovery was not significantly better until day 21. The BBB scores of amiloride treated SCI rats were significantly higher than those for vehicle-treated SCI animals on days 21, 28, and 35 (Fig. 1A), with a maximum difference of ~2 BBB units (from an average BBB score of ~11 to ~13), which indicated an improved coordination between forelimbs and hindlimbs in amiloride-treated animals.

FIG. 1. (A) All rats (male Sprague-Dawley) were contused at T10 using an Infinite Horizon device and a force of 150 kDynes, 1 sec dwell time. All surgical procedures are described in Nesic et al., (2008). Naïve animals ($n=6$) were not subjected to any part of the surgical or postsurgical care protocols. All procedures complied with the recommendations in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the University of Texas Medical Branch Animal Care and Use Committee. Hindlimb movement was assessed using the Basso, Beattie, and Bresnahan Scale, also known as the BBB scale (Basso et al., 1995). BBB scores were collected daily the first 14 days after injury, and once weekly thereafter. The BBB scale assigns values to the left and right hindlimbs corresponding to their locomotor ability. Combined left and right hindlimb BBB locomotor scores of SCI+amiloride (AML) ($n=6$) and SCI+vehicle (V) ($n=6$) animals are shown (mean $\pm$ SE; * $p < 0.05$; repeated measure two-way ANOVA without replication; SPSS statistical package, version 11.0). Animals were weighed weekly to adjust the administration of amiloride to account for weight increases over time for each individual animal. (B) Recovery of bladder function in vehicle- and amiloride-treated SCI rats. SCI rats cannot spontaneously empty their bladders during the first days after injury, and therefore the care of SCI rats includes manual emptying of their bladders twice a day. However, SCI rats over time recover their ability to spontaneously empty their bladders, and that was presented in this graph. Therefore, in our experiment, spontaneous emptying of bladders in both experimental groups was restored at ~ 7 days after SCI. Mean $\pm$ SE.

The bladder function of both vehicle- and amiloride-treated rats returned at day 7 or thereabouts (Fig. 1B), indicating that amiloride did not impair the recovery of bladder function. To further assess possible side effects of amiloride, we also evaluated animal weight changes over time. Given that amiloride is a mild diuretic, it can cause modest weight loss. We found that amiloride-treated rats did not gain as much weight over 35 days after SCI as did vehicle-treated SCI rats. However, these small weight differences ($\sim 8\%$) were expected and were not worrisome.

We also conducted an experiment in which amiloride was administered at 10 mg/kg i.p. starting 35 days after SCI. However, such late administration of amiloride ($n=8$ per group) did not produce an improvement in locomotor recovery.

Amiloride Reduces Myelin Loss

Motor recovery after SCI depends upon axonal sparing (Basso 2000; Basso et al., 2002), remyelination of surviving axons (Cao et al., 2010), and plastic reorganization of the properly myelinated/functional surviving axons to form new spinal circuits (Steencken et al., 2009; Bareyre et al., 2004; Edgerton et al., 2004).

Given that the beneficial effects of amiloride were delayed to 2–3 weeks after SCI, we hypothesized that amiloride affects processes of axonal de/remyelination, which take place in that time period after SCI (Totoiu and Keirstead, 2005). To assess changes in myelin levels after SCI and amiloride treatment, we measured protein levels of myelin oligodendrocyte glycoprotein (MOG), a membrane protein expressed on the oligodendrocyte cell surface and on the outermost surface of myelin sheaths. Although changes in myelin levels after SCI have been extensively analyzed; changes in MOG levels after SCI and their correlation with locomotor recovery have not previously been reported. As is shown in Figure 2A, MOG levels were modestly decreased in the acute phase post-SCI (1 and 3 days after SCI), reflecting primarily an immediate loss of myelinated axons and oligodendrocytes at the site of injury. However, at 7 days after SCI, MOG levels were markedly more reduced ($\sim 90\%$; Fig. 2A), reflecting the delayed loss of oligodendrocytes and the resulting demyelination of surviving axons, consistent with the findings of Wrathall and associates (1998). We also found similar decreases in MOG levels at 21 or 35 days after SCI.

We also found that MOG levels closely correlated with the extent of hindlimb locomotor impairment after SCI, as is shown in Figure 2B. We analyzed MOG levels in three groups of SCI rats that were classified according to their distinctly different BBB scores (≥ 4 BBB units difference): 1) SCI rats with an average 21-day BBB score of 8 ± 0 ($n=3$); 2) SCI rats with an average 21-day BBB score of 12.5 ± 0.3 ($n=3$); and 3) SCI rats with an average 21-day BBB score of 18 ± 0 ($n=3$). As is shown in Figure 2B, there was a positive association between different MOG protein levels and locomotor recovery after SCI, not only at 21 days, but also at 56 days after SCI, with similar MOG levels being correlated with the same extent of motor recovery at both time points, indicating that the same association between MOG levels and BBB scores persisted beyond 21 days after SCI (at least until 56 days after SCI). Furthermore, it appears that even modest increases in MOG levels (by $\sim 12\%$) were associated with marked improvements in

locomotor recovery (BBB scores changed from 8 to 12), although nearly complete recovery of hind limb motor function (BBB scores of ~ 18) appears to require more substantial preservation of MOG ($\sim 50\%$).

We found that amiloride treatment in SCI rats (the same rats as in Figure 1) significantly increased MOG levels by 19% at the site of injury (T10), 35 days after SCI ($p=0.0304$; $n=6$ per group) (Fig. 2C, D). Based on our data presented in Figure 2A and B, we conclude that amiloride probably affected the delayed myelin loss that occurs in the first 7 days after SCI; and that the 19% increase in myelin content 35 days after SCI could explain the two-point BBB score improvement in locomotor recovery (Fig. 1).

Our late amiloride intervention experiment, in which amiloride treatment began 35 days after SCI and continued

FIG. 2. (A) A representative Western blot showing myelin oligodendrocyte glycoprotein (MOG) monomer band at ~ 23 kD from the site of injury (T10), 3 days and 7 days after SCI. Western blot methods are described in detail in Nesic et al., 2005, 2006, 2008, 2010. As a loading control in all our Western blots we use β -actin (presented here as a band of ~ 40 kD). MOG antibody was purchased from Abcam (ab28766, dilution: 1 μ g/mL). **Bar graph:** We also analyzed MOG protein levels at different time points after SCI, 1 ($n=5$), 3 ($n=5$), 7 ($n=3$), 21 ($n=5$), and 35 days ($n=6$) after SCI (mean $\pm$ SE). The 23 kD band was quantified and normalized, first to β -actin levels, and then to uninjured values (uninjured signal intensity=100%). β -actin antibody was purchased from Sigma (A5441, dilution 1:80,000). (B) We analyzed MOG levels in SCI rats with distinctly different levels of motor recovery 21 days or 56 days post-SCI as indicated by the BBB scores. We have chosen to analyze MOG levels in SCI rats whose BBB scores are different by ≥ 4 BBB units, given that we did not expect that smaller differences in BBB scores would have been translated into measurable alterations in MOG levels, considering inter-individual variations in MOG levels (see MOG bands for SCI rats with the same BBB scores). Higher MOG levels at the lesion site (T10) were associated with better hindlimb locomotor recovery at both time points after SCI: a positive association between MOG levels and motor recovery was nearly unchanged from 21 to 56 days after SCI. **Bar graph:** MOG monomer band intensities were quantified and normalized, first to β -actin levels, and then to uninjured values (uninjured signal intensity=100%). The corresponding BBB scores are written above each bar (mean $\pm$ SE). (C) We analyzed MOG protein levels in three groups of rats: naive ($n=5$), SCI rats treated with vehicle ($n=6$), and SCI rats treated with amiloride (AML) ($n=6$). Their BBB scores are shown in Figure 1. MOG levels were markedly decreased at the lesion site 35 days after moderate SCI, whereas amiloride treatment significantly increased the MOG monomer and dimer levels (dimer 46kD band, believed to be the active MOG form; Clements et al., 2003). MOG dimer levels paralleled changes in monomer bands in all our experiments. (D) Quantitative analyses of the monomer band showed that MOG levels at T10 were significantly higher in amiloride-treated SCI rats (mean $\pm$ SE $*p=0.03$ t -test). Although MOG levels at L4/5 decreased $\sim 40\%$ after SCI, they were not significantly different from uninjured levels. This was primarily because of inter-individual variations, which also obscured a modest increase in MOG in amiloride-treated SCI rats. Therefore, it is possible that amiloride also affected myelin loss in lumbar segments that directly control hindlimb locomotion, but this remains to be analyzed further.

until 70 days after SCI, did not improve locomotor recovery; therefore amiloride intervention appears to be most effective between 24 h and 35 days after SCI. If the beneficial outcome resulted only from the amiloride effect on myelin sparing, then amiloride administration starting later than 7 days after SCI would not be effective. This is because the processes underlying significant MOG loss were mostly finalized at 7 days after SCI or thereabouts, as MOG level decreases were similar at 7, 21, and 35 days after SCI (Fig. 2A). However, other processes relevant to motor recovery that occur beyond 7 days after SCI, such as altered axonal conductivity (Arvanian et al., 2009; Blight et al., 1983), can also be affected by amiloride because of its potent modulation of sodium and calcium transport mechanisms (as described in the Introduction). Therefore, it is possible that amiloride intervention should last longer than 7 days after SCI, but the optimal therapeutic window for amiloride intervention between 24 h and 35 days after SCI remains to be determined.

How can amiloride preserve myelin after SCI? It is possible that amiloride protects oligodendrocytes, whose prolonged death over weeks after SCI is well documented (Wrathall et al., 1998), and is consistent with our data (Fig. 2A). However, the mechanisms that underlie delayed oligodendrocyte death are poorly understood, although apoptosis triggered by hypoxia has been documented (Dewar et al., 2003). It has been shown that brain oligodendrocytes express ASICs (Feldman et al., 2008) and NHEs (Ro and Carson, 2004), and that spinal cord oligodendrocytes express NCX (Chen et al., 2007). Therefore, inhibition of these amiloride targets in oligodendrocytes that are exposed to damaging hypoxic conditions in injured spinal cords may underlie the effect of amiloride on MOG. It is also possible that oligodendrocyte expression of amiloride targets changes over time after SCI, thus rendering surviving or newly generated mature oligodendrocytes more susceptible to hypoxia-induced apoptosis in the sub-chronic post-SCI phase. However, possible involvement of amiloride targets in delayed oligodendrocyte apoptosis after SCI remains to be determined.

Alternatively, oligodendrocyte progenitor cells generated during the sub-chronic phase after SCI to replace lost oligodendrocytes (Sellers et al., 2009) can also express amiloride targets and thus become vulnerable to hypoxic conditions after SCI. Feldman and associates (2008) found that the oligodendrocyte progenitor cells express high levels of ASICs; therefore protection of these cells can also contribute to the delayed beneficial effects of amiloride. Oligodendrocyte progenitor cells also express NHEs (Boussof and Gaillard, 2000); and probably express an NCX as well (Liu et al., 1997). Therefore, amiloride may affect processes of remyelination by newly formed oligodendrocytes, and therefore myelin levels after SCI, by sparing oligodendrocyte progenitor cells. However, this remains to be investigated in our future experiments.

We did not find a significant effect of amiloride on the loss of neuronal class III β -tubulin (TUB-1)-labeled axons, neuronal nuclei (NeuN)-labeled neurons, or on glial activation. Microglial activation was measured with Iba-1 (see Carlton et al., 2009), and astrocytic activation was measured with glial fibrillary acidic protein (GFAP) and aquaporin 4 (AQP4), not shown. All Western blot protein measurements were per-

formed on the lumbar segments and lesion site, 35 days after SCI. For full methods regarding these markers see studies by Nesic and associates (2005, 2006, 2008, and 2010).

Although our data show that the effect of amiloride on myelin levels (Fig. 2C and D) may be sufficient to explain improvements in locomotor recovery, other processes relevant to functional recovery after SCI that could also be affected by amiloride cannot be ruled out. For example, it is conceivable that amiloride may also modulate angiogenesis after SCI (Alliegro et al., 1993; Knoll et al., 1999; Mitternig-Grosse et al., 2006), and altering angiogenesis can critically affect functional recovery after SCI, as is elegantly shown by Han and associates (2010).

More effective improvements in locomotor recovery after SCI maybe achieved with higher amiloride doses (> 10 mg/kg). However, higher amiloride doses have potentially harmful systemic effects, because amiloride also blocks the epithelial sodium channels in the tubules of the kidney (for a review see Hamm et al., 2010). Although amiloride is a weak diuretic (it inhibits $< 10\%$ of sodium reabsorption by kidneys), high amiloride doses can induce potentially dangerous hyperkalemia. Because of these effects, the LD₅₀ value for amiloride is between 36 and 86 mg/kg in normal rats (the amiloride LD₅₀ value in humans is not known). However, it can be expected that the LD₅₀ dose of amiloride may be lower in SCI rats, given the impairment of kidney function after SCI (Ruffion et al., 2007; Samson and Cardenas, 2007; Vaziri et al., 1984). Therefore, higher amiloride doses (> 10 mg/kg) may be tested only if appropriate methods are used to avoid the systemic effects of amiloride. This could be achieved by using liposome-encapsulated amiloride, with liposomes that are designed to avoid the kidneys using an approach similar to the reversible masking method described by Shi and associates (2010). It has already been shown that systemically administered liposome-encapsulated amiloride does not increase serum potassium levels, in contrast to systemically administered free amiloride (Ali et al., 2007).

Conclusion

Taken together, our data show that amiloride, a drug approved by the United States Food and Drug Administration that penetrates the blood-brain barrier, may have clinically relevant therapeutic potential in SCI because it significantly improved locomotor recovery, and because it is the only pharmacological intervention that has shown myelin-sparing effects after SCI.

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Author Disclosure Statement

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