

Na-K-Cl Cotransporter-1 as a Regulator of Manganese-induced Astrocyte Swelling

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Abstract

Astrocyte swelling leads to brain edema, intracranial pressure, brain herniation and acute liver failure (fulminant hepatic failure) which is the major cause of death in this condition. Manganese has been strongly implicated as an important factor in astrocyte swelling. Manganese in excess is neurotoxic and causes a CNS disorder that resembles Parkinson's disease (manganism). Manganese highly accumulates in astrocytes, which renders these cells more vulnerable to its toxicity. In addition to manganism, increased brain levels of manganese have been found in hepatic encephalopathy. Manganese is known to cause cell swelling in cultured astrocytes, although the means by which this occurs has not been fully elucidated. A disturbance in one or more of these systems may result in loss of ion homeostasis and cell swelling. In particular, activation of the Na-K-Cl cotransporter-1 (NKCC1) has been shown to be involved in cell swelling in several neurological disorders. We therefore examined the effect of manganese on NKCC activity and its potential role in the swelling of astrocytes. Cultured astrocytes were exposed to manganese (50 μ M), and NKCC activity was measured. Manganese increased NKCC activity at 24 h. Inhibition of this activity by bumetanide diminished manganese-induced astrocyte swelling. Manganese (Mn) also increased total as well as phosphorylated NKCC1. These results suggest that activation of NKCC1 is an important factor in the mediation of astrocyte swelling by manganese and that such activation appears to be mediated by NKCC1 abundance.

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INTRODUCTION

Acute liver failure (fulminant hepatic failure) is associated with cerebral edema, increased intracranial pressure, herniation, and death (Vaquero *et al.*, 2003). Although the pathogenetic mechanisms for brain edema are still not clear, manganese has been strongly implicated as an important etiological factor (Rama Rao *et al.*, 2007), and astrocyte swelling has been implicated as a major process responsible for the edema (cytotoxic edema) (Martinez, 1968; Norenberg, 1977; Norenberg, 2001; Traber *et al.*, 1989). Mechanisms mediating the astrocyte swelling and the subsequent brain edema in fulminant hepatic failure remain poorly understood.

Manganese is an essential trace metal and is an integral component of key enzymes such as glutamine synthetase (Wedler *et al.*, 1982) and mitochondrial

superoxide dismutase (McCard, 1976). However, excess deposition of manganese in the CNS leads to neurological abnormalities (manganism) which is associated with astrocyte swelling (Rama Rao *et al.*, 2007). Manganese in the brain is preferentially deposited in astrocytes because of the presence of high capacity transporters in these cells (Aschner *et al.*, 1992; Aschner *et al.*, 1999). Such preferential accumulation suggests that astrocytes may be more vulnerable to manganese toxicity than other neural cells. Consistent with this possibility, primate models of manganese toxicity have shown astrocytic pathological alterations (Olanow *et al.*, 1996; Pentschew *et al.*, 1963).

Ion channels, exchangers, and transporters represent important factors in the mechanism of cell volume regulation (for reviews, see Refs. Hoffmann and Dunham,

1995; Mobasher *et al.*, 1998; O'Neill, 1999; Liang *et al.*, 2007). Structural and functional changes in these systems may result in the loss of ion homeostasis (for a review, see Ref. Kintner *et al.*, 2007) and the subsequent accumulation of intracellular water. These ion transporters and exchangers include the Na-K-Cl cotransporter (NKCC), volume-sensitive osmolyte anion channels, $\text{Na}^+/\text{Ca}^{2+}$ exchanger, the Na^+/H^+ exchanger, and the nonselective cation channel ($\text{NC}_{\text{Ca-ATP}}$) (Liang *et al.*, 2007). The NKCC1, in particular, has been shown to play an important role in the mediation of cell swelling/brain edema (for a review, see Ref. Chen and Sun, 2005). NKCC cotransporters are a class of membrane proteins that transport Na^+ , K^+ , and Cl^- ions into and out of a wide variety of epithelial and nonepithelial cells and are part of a super family of cation-chloride cotransporters (Haas and Forbush, 2000). Two isoforms of NKCC have been identified (NKCC1 and NKCC2) (Xu *et al.*, 1994; Haas and Forbush, 2000). NKCC1 is present in many cell types, including astrocytes (Tas *et al.*, 1986; Tas *et al.*, 1987; Walz, 1992; Plotkin *et al.*, 1997; Su *et al.*, 2000; Yan *et al.*, 2001). The NKCC2 isoform is localized exclusively to the kidney (Ecelbarger *et al.*, 1996; Payne and Forbush, 1994). NKCC1 activation was shown to contribute to astrocyte swelling/brain edema in ischemia and to brain edema after traumatic brain injury (MacVicar *et al.*, 2002; Su *et al.*, 2002a; Su *et al.*, 2002b; Staub *et al.*, 1994; Lu *et al.*, 2006; Lu *et al.*, 2007). Its role in astrocyte swelling after manganese treatment and in the brain edema in fulminant hepatic failure, however, is not known.

This study investigated whether NKCC activation occurs in manganese-treated astrocyte cultures and whether such activation contributes to astrocyte swelling. Our findings demonstrate that manganese increased NKCC activity, which was associated with an increase in total and phosphorylated NKCC1 protein expression. Blocking NKCC activity with bumetanide attenuated manganese-induced astrocyte swelling. Altogether, our findings suggest that activation of NKCC plays an important role in the mediation of manganese-induced astrocyte swelling and possibly in the brain edema associated with fulminant hepatic failure.

MATERIALS AND METHODS

Astrocyte Cultures

Astrocyte cultures were prepared from brains of 1-2 day old rat pups by the method of Ducis *et al.* (1990). Briefly, cerebral cortices were freed of meninges, minced, dissociated by trituration and vortexing, passed through sterile nylon sieves, placed in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, and fetal bovine serum, and incubated at 37 °C in a humidified chamber provided with 5% CO_2 and 95% air. After 10 days in culture, bovine serum was replaced with 10% horse serum. After 14 days, cultures were treated and maintained with dibutyryl-cAMP (Sigma) so as to enhance cell differentiation (Juurlink and Hertz, 1985). Cultures consisted of at least 98% astrocytes, as determined by glial fibrillary acidic protein and glutamine synthetase immunocytochemistry. The remaining cells consisted of microglia. Experiments were carried out in 3-4 week old cells. Procedures followed guidelines established by the National Institutes of Health Guide for the care and use of laboratory animals and were approved by the local animal care committee (HSKOP/IAEC, CLEAR/2010-11/1-11).

NKCC Activity

NKCC activity was measured as the bumetanide-sensitive K^+ influx, using ^{86}Rb as a tracer for K^+ by a modification of the method of Sun and Murali (1999). Briefly, primary astrocyte cultures were treated once with a pathophysiological concentration of manganese (50 μM). At the end of the treatment (24 h), cultures were equilibrated and preincubated with or without bumetanide (50 μM) for 15–30 min at 37 °C in an isotonic HEPES-minimal essential medium (140mM NaCl, 5.36 mM KCl, 0.81 mM MgSO_4 , 1.27 mM CaCl_2 , 0.44 mM KH_2PO_4 , 0.33 mM Na_2HPO_4 , 5.55 mM glucose, and 20 mM HEPES (300 mosM)). Cultures were then exposed to 1 $\mu\text{Ci/ml}$ ^{86}Rb for 3 min and subsequently rinsed with ice-cold 0.1 M MgCl_2 . Cells were then extracted in 1% SDS, and the radioactivity was analyzed by liquid scintillation. The ^{86}Rb influx rate was calculated by subtracting the influx (with bumetanide) from total influx (without bumetanide) and expressed as nmol of $^{86}\text{Rb}/\text{mg}$ of protein/min. Quadruplicate determinations were obtained throughout the study, and the protein content was determined employing the BCA assay (Pierce).

Cell Volume Determination

Cell volume was estimated by measuring the intracellular water space by the method of Kletzien *et al.* (1975), as modified by Kimelberg (1987) and Bender and Norenberg (1998). Briefly, 1 mM 3-O-methylglucose and 0.5 $\mu\text{Ci/ml}$ ^3H -labeled 3-O-methylglucose were added to the culture 6 h before the volume assay. At the end of the incubation period, culture medium was aspirated, and an aliquot was saved for radioactivity determination. Cells were rapidly washed six times with ice-cold buffer containing 229 mM sucrose, 1 mM Tris nitrate, 0.5 mM calcium nitrate, and 0.1 mM phloretin, pH 7.4. Cells were then harvested into 0.5 ml of 1 N sodium hydroxide. Radioactivity in the cell extracts and medium were determined, and an aliquot of the cell extract was used for protein estimation (BCA method). Values were normalized to protein level, and the cell volume was expressed as $\mu\text{l}/\text{mg}$ of protein.

Western Immunoblots

Astrocyte cultures were solubilized in lysis buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, phosphatase inhibitors (Sigma), and a protease inhibitor mixture (Roche Applied Science), and protein levels were measured by the BCA method. Equal amounts of protein were subjected to gel electrophoresis, as previously described (Jayakumar *et al.*, 2006), and transferred to nitrocellulose membranes. Following blocking with nonfat dry milk, membranes were incubated with respective antibodies. Total NKCC1 antibody was purchased from Chemicon International (Temecula, CA). Primary antibodies to detect phosphorylated (R5) and total NKCC1 were used at 1:1000. Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories) were used at 1:1000. Optical density of the bands were determined with the Chemi-Imager (Alpha Innotech, San Leandro, CA) digital imaging system, and the results were quantified with the Sigma Scan Pro (Jandel Scientific, San Jose, CA) program as a proportion of the signal of a housekeeping protein band (β -actin).

Each experimental group consisted of 4-5 culture dishes/experiment for each time point studied in the cell

swelling experiments. At least 2-4 plates were used for Western blot analysis. All experiments were performed from 4-7 separate seedings. Data were subjected to ANOVA, followed by Tukey's *post hoc* comparisons. At each time point, the experimental cultures were compared with their respective controls.

RESULTS

Cell Volume Increased at Different Concentration of Manganese for 24 Hours

Manganese was treated at different concentrations for 24 h. The cell volume increased at 20µM concentration and peaked at 50µM and persisted up to 100µM concentrations (Figure 1).

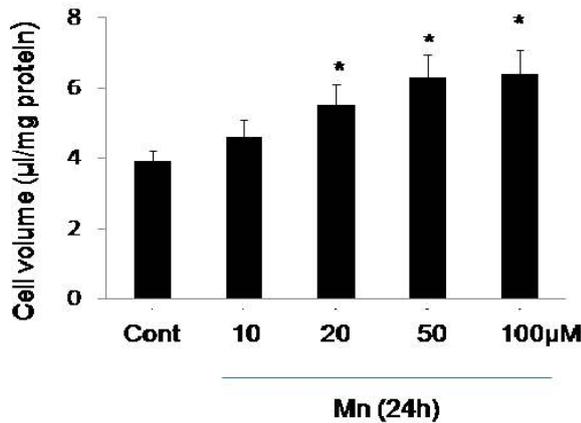


Figure 1: Manganese induced cell volume at different concentrations. Cultured astrocytes were exposed to different concentrations of manganese (10, 20, 50 and 100 µM) for 24 hours and the cell volume was measured. Manganese significantly increased cell volume at 20, 50 and 100 µM. ANOVA, n=5. *p<0.05 vs. control. Error bars represent mean ± SEM.

Cell Volume Increased at Different Time Period of Manganese Treatment (50 µM)

Manganese was treated for different time period with 50µM concentrations. The cell volume increased at 6 h and persisted up to 48 h with a peak increase at 24 h (Figure 2).

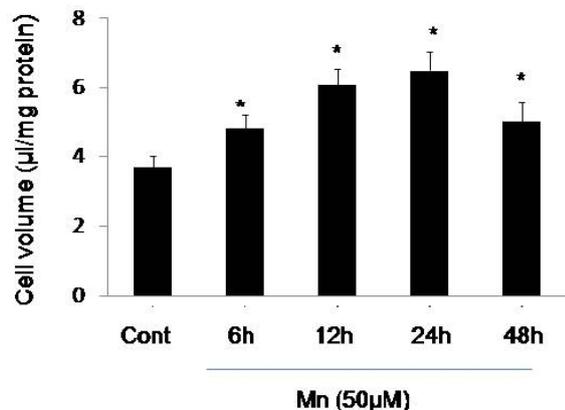


Figure 2: Manganese induced cell volume at different time periods. Astrocytes were exposed to manganese (50 µM) for different time periods (6, 12, 24 and 48 h). Manganese significantly increased the cell volume at all time points studied. However, there was a decline after 48 h. ANOVA, n=4. *p<0.05 vs. control. Error bars represent mean ± SEM.

NKCC Activation by Manganese in Cultured Astrocytes

Manganese significantly increased NKCC activity. Initial increase was observed at 12 h, which persisted up to 24 h, with a peak increase occurring at 24 h (Figure 3).

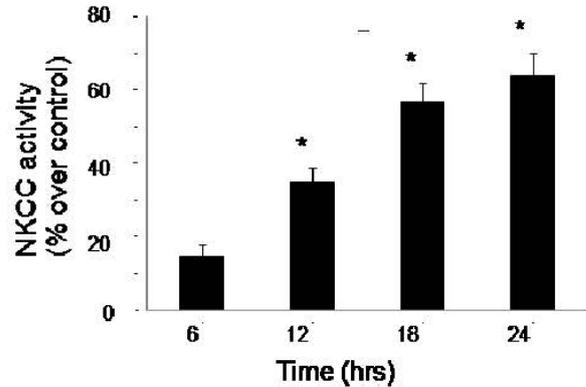


Figure 3: Manganese induced NKCC activity. Cultured astrocytes were exposed to 50 µM Mn for different time periods (1-24 h) and the bumetanide-sensitive NKCC activity was measured. Manganese significantly increased NKCC activity at 12, 18 and 24 h. ANOVA, n=5. *p<0.05 vs. control. Error bars represent mean ± SEM.

Treatment of Bumetanide Attenuated Manganese-Induced Astrocyte Swelling

Astrocyte cultures were exposed to manganese for 24 h with or without bumetanide (50 µM), and cell volume was determined. The intracellular water space of control primary astrocytes culture was 4.2 ± 0.1 µl/mg proteins. Cultures exposed to manganese showed a significant increase in cell volume (39% as compared to control, p<0.05). Cell swelling was significantly attenuated by treatment with 50 µM bumetanide (66%, p<0.05 vs. control) (Figure 4).

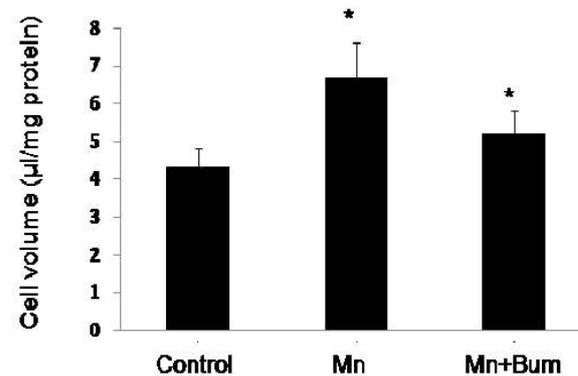


Figure 4: Effect of Bumetanide on astrocytes swelling. Cultured astrocytes exposed to 50 µM Mn significantly increased cell swelling (54%) at 24 h. Treatment with bumetanide (BUM, 50 µM) significantly diminished such swelling by 66%. ANOVA, n=5. *p<0.05 vs. control. Error bars represent mean ± SEM.

Manganese Increased NKCC1 Protein Expression

Cultures exposed to manganese significantly increased the total NKCC1 protein level as measured by Western blots. The initial increase was observed at 6 h and persisted for up to 24 h (Figure 5).

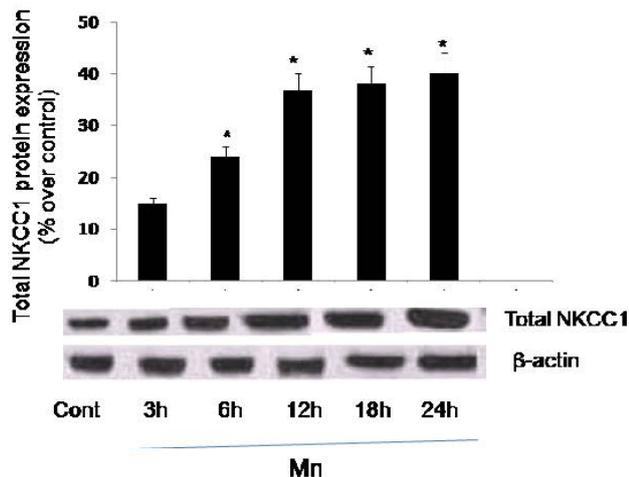


Figure 5: Effect of Manganese on total NKCC1 protein level. Western blots show a significant increase in total NKCC1 protein level when cultures were exposed to 50 μ M Mn. NKCC1 levels were normalized against β -actin. ANOVA, $n=5$. * $p<0.05$ vs. control. Error bars represent mean \pm SEM.

Manganese Increased the Phosphorylation of NKCC1

Manganese also significantly increased the phosphorylation of NKCC1 at all time points (from 6 h to 24 h). The cultures were treated with 50 μ M of Manganese at all time points (Figure 6).

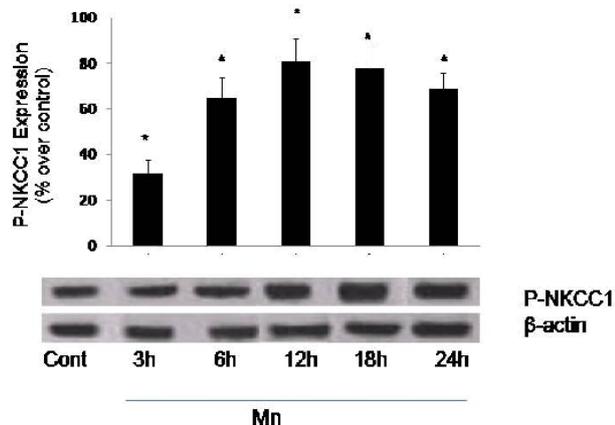


Figure 6: Effect of Manganese on Phosphorylated NKCC1 Expression. Western blots show a significant increase in phosphorylated NKCC1 (p-NKCC1) level when cultures were exposed to 50 μ M Manganese. Phospho-NKCC1 levels were normalized against β -actin. ANOVA, $n=6$. * $p<0.05$ vs. control. Error bars represent mean \pm SEM.

DISCUSSION

We have studied the role of Na-K-Cl cotransporter-1 in the manganese induced astrocyte swelling. This study demonstrates the involvement of the ion transporter protein NKCC1 in the mechanism of manganese induced astrocyte swelling. Cultured astrocytes exposed to manganese increased the cell volume with the increase in time and concentrations. Manganese also increased the bumetanide sensitive NKCC activity as well as total and phosphorylated (activated) NKCC1. Blocking NKCC activation with bumetanide diminished manganese-induced cell swelling. These findings suggest that manganese-induced activation of NKCC1 is an important

factor in the mediation of cell swelling in cultured astrocytes, and that such activation appears to be mediated by phosphorylation of NKCC1.

NKCC is involved in the regulation of several cellular functions, including ion transport across secretory and absorptive epithelia (for reviews, see ref. Haas, 1994), NH_4^+ transport (Nagaraja and Brooks, 1998), maintenance and regulation of cell volume and ion gradients (for reviews see ref. Kaplan *et al.*, 1996). The electroneutral NKCC protein transports Na^+ , K^+ , and Cl^- into cells under both physiological and pathophysiological conditions with a stoichiometry of $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ (for review, see ref. Haas and Forbush, 1998). NKCC can be inhibited by either bumetanide or furosemide (Kimmelberg and Frangakis, 1985; Russell, 2000). NKCC1 is present in many cell types, including astrocytes (Yan *et al.*, 2001; Hoppe and Kettenmann, 1989; Wang *et al.*, 2003; Alvarez-Leefmans, 2001). However, the NKCC2 isoform is localized exclusively to the kidney (Ecelbarger *et al.*, 1996; Payne and Forbush, 1994). Activation of NKCC has been shown to cause brain edema in several neurological disorders. A recent study showed that administration of bumetanide, either prior to or during ischemia, significantly reduces brain edema in rats (Yan *et al.*, 2001). Activation of NKCC1 was shown to contribute to brain edema after traumatic brain injury (Staub *et al.*, 1994; Lu *et al.*, 2006; Lu *et al.*, 2007).

NKCC1 plays an important role in cell volume regulation in brain astrocytes (Su *et al.*, 2002a; Su *et al.*, 2002b; Chen and Sun, 2005). The cotransporter activity in astrocytes is significantly stimulated in response to high K^+ in a Ca^{2+} dependent manner (Su *et al.*, 2000). Stimulation of NKCC1 by high K^+ results in cell swelling through a net increase of intracellular Na^+ , K^+ , Cl^- and accompanying water. Recently, Su *et al.* (2002) showed that inhibition of NKCC by bumetanide abolishes high extracellular K^+ -induced swelling and intracellular Cl^- accumulation in rat cortical astrocytes. These authors also showed that astrocytes from NKCC1 cotransporter-null mice exhibit an absence of swelling. Consistent with these findings, our study shows that astrocyte cultures exposed to manganese caused a significant increase in bumetanide-sensitive NKCC activity and that blocking such activity significantly reduced manganese-induced astrocyte swelling. These findings indicate the involvement of NKCC1 in the mechanism of astrocyte swelling following manganese exposure. Activation of NKCC1 results in the accumulation of sodium inside the cell and such increase in $[\text{Na}^+]_i$ may cause cellular swelling (Xie *et al.*, 1994). An increase in $[\text{Na}^+]_i$ concentration due to increased NKCC1 activity was observed in rat cortical astrocytes after chemical hypoxia and in rat spinal cord astrocytes after glucose deprivation (Rose *et al.*, 1998); and in mouse cortical astrocytes after ischemia (Silver *et al.*, 1997). NKCC1-mediated intracellular Na^+ accumulation and cell swelling was also observed in astrocytes exposed to oxygen and glucose deprivation/reoxygenation, and such increase was diminished by bumetanide (Lenart *et al.*, 2004).

Phosphorylation of NKCC1 has been shown to activate its activity (Lyte and Forbush, 1992). Phosphorylation of Thr184/Thr189 correlates with NKCC1 activation in epithelial cells (Darman and Forbush, 2002).

Such a correlation has also been found in cultured cortical neurons (Schomberg *et al.*, 2003). Our study demonstrates that manganese results in the phosphorylation (activation) of NKCC1. We also found a significant increase in total NKCC1 protein level after manganese treatment. Studies have shown that reduced NKCC1 protein expression diminish NKCC activity when bovine brain microvessel endothelial cells are exposed to hypoxia/aglycemia (Abbruscato *et al.*, 2004). Taken together, these studies indicate that manganese causes an increase in both total and phosphorylated NKCC1 and that such increase was associated with its enhanced activity.

CONCLUSION

Our study reveals that NKCC1 is involved in the mediation of manganese-induced astrocyte swelling, and that phosphorylation of NKCC1 contributes to such activation. Targeting NKCC1 may provide a useful strategy in the treatment of the brain edema associated with fulminant hepatic failure.

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