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Rapid communication

Magnesium sulfate protects oligodendrocyte lineage cells in a rat cell-culture model of hypoxic–ischemic injury

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ABSTRACT

Hypoxic–ischemic (HI) brain injury in newborns results in serious damage. Magnesium sulfate has been clinically used as a cyto-protective agent against HI brain injury in newborns in some countries, including Japan. However, it is not clear how magnesium exerts this effect and how it acts on the individual types of cells within the newborn brain. In this study, we exposed cultured rat oligodendrocyte precursor cells to magnesium sulfate during the period when they differentiate into oligodendrocytes, and showed that magnesium-exposed oligodendrocytes exhibited more resistance to HI injury. Our data may support the use of magnesium sulfate in the clinical setting.

White matter injury after hypoxic–ischemic (HI) injury results in serious damage to the developing brain. Many affected infants suffer from severe neurological deficits and lifelong handicaps. Therefore, therapy to protect the white matter and reduce the injury is urgently needed. In seeking therapeutic agents that can provide protection to the vulnerable developing brain, magnesium sulfate has been examined for its potential. Although still controversial, magnesium is one of the supportive drugs used in newborn HI injury, often in conjunction with therapeutic hypothermia (Shea and Palanisamy, 2015). However, effects of magnesium in newborn hypoxia and/or ischemia are not fully understood, and it is important to evaluate whether and how magnesium can protect developing brains against hypoxic stress. In this study, therefore, we examined the effects of magnesium sulfate on oligodendrocyte lineage cells (oligodendrocytes and oligodendrocyte precursor cells), which constitute the major cell types in cerebral white matter.

All experiments were performed following institutionally approved protocol by Massachusetts General Hospital Subcommittee on Research Animal Care, and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Primary oligodendrocyte precursor cells (OPCs) were isolated from neonatal rat brain cortex as previously described (Arai and Lo, 2009). For proliferation, OPCs were cultured in Neurobasal media containing 1% penicillin/streptomycin, 2 mM L-glutamine, 10 ng/ml basic fibroblast growth factor, 10 ng/ml platelet-derived growth factor-AA, and 2% B27 supplement. For differentiation of OPCs into mature oligodendrocytes, culture media was switched to differentiation media (Dulbecco's Modified Eagle's Medium (DMEM) containing 1% penicillin/streptomycin, 10 ng/ml ciliary neurotrophic factor, 15 nM triiodo-L-thyronine, and 2% B27 supplement).

Maturation of OPCs was assessed on the seventh day after the start of differentiation. Western blots using anti-myelin basic protein (MBP) and glutathione-S-transferase (GST) pi, which are markers for mature oligodendrocytes, and PDGF receptor-α (PDGFR-α), a marker for OPCs, were done to confirm the maturation state of the cells. Western blot of cell lysates was carried out using mouse monoclonal antibodies to MBP (1:500 dilution, Thermo scientific), rabbit polyclonal antibodies to GST-pi (1:1000 dilution, MBL international), rabbit polyclonal antibodies to PDGFR-α (1:1000 dilution, Santa Cruz) and mouse monoclonal antibodies to β-actin (1:10,000 dilution, Sigma Aldrich). OPC maturation was also assessed by quantitative real-time PCR (QRT-PCR) on the third day after switching the culture media. RNA was isolated with RNeasy Plus Mini kit (QIAGEN, Hilden, Germany), and first-strand cDNA was synthesized with PrimeScript RT reagent (Takara-Clonetech). QRT-PCR was performed using SYBR Premix

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Ex Taq II(Takara-Clonetech) and analyzed with Fast real time system 7500 (Applied Biosystems). Expression levels were measured relative to GAPDH as internal control. The sequences of primers used in this study are as follows—5′ ttgactccatcgggcgcttcttta-3′ for rat MBP forward; 5′ ttcatcttgggtc ctctgcgactt-3′ for rat MBP reverse. These markers (PDGFR-α for OPCs, MBP/GST-pi for mature oligodendrocytes) are now relatively well accepted in the literature (Galvao et al., 2014; Han et al., 2015), and we have been using them consistently in our previous studies (Maki et al., 2015; Miyamoto et al., 2015).

Oligodendrocytes matured in the presence of magnesium sulfate or vehicle (control oligodendrocytes) both underwent oxygen/glucose deprivation (OGD) in a rat cell-culture model of HI injury. OGD experiments were conducted as previously described (Arai and Lo, 2009). During the OGD period (2-h), culture media was changed to DMEM that contained no glucose, and switched back to OPC differentiation media with added magnesium sulfate or vehicle after OGD. After 24 h of re-oxygenation, cell viability was evaluated by WST assay (Cell counting kit, Dojindo) or direct cell counting in a blinded manner. All results presented in this study were expressed as mean ± S.D. Statistical significance was evaluated using Mann–Whitney *U* test to compare between two groups, and Kruskal-Wallis test for multiple comparisons. A p value of <0.05 was considered to be statistically significant.

During the period of OPC maturation in vitro, primary rat OPC cultures were exposed to 5 mM magnesium sulfate. This magnesium dose was compatible with previous studies, which showed beneficial effects of magnesium for neural stem cells and endothelial cells in vitro (Lapidos et al., 2001; Vennemeyer et al., 2014). In the clinical setting, the physiological serum magnesium concentration is around 1 mM, but may increase to 3 mM after magnesium administration (Westermaier et al., 2013). In addition, the serum magnesium concentration in asphyxiated newborns who received magnesium therapy was reported to be 1.4–2.8 mM (Bhat et al., 2009). In our cell culture system, OPCs started their differentiation process once their culture media was switched to the differentiation media. In fact, even in the vehicle group, cells showed oligodendrocyte-like shape on day 7 after starting OPC differentiation (Fig. 1a—left). However, when OPCs were exposed to 5 mM magnesium sulfate for 7 days during the time period of differentiation, cells exhibited more matured oligodendrocytes with spider's web-like processes (Fig. 1a—right). On the other hand, magnesium sulfate did not affect cell survival (Fig. 1b) and cell number (Fig. 1c). Since magnesium sulfate changed cell morphology, we then assessed whether magnesium sulfate promoted the maturation process of OPCs. As expected, protein expression of PDGFR-α (OPC marker) decreased in the magnesium sulfate-treated group (Fig. 2a–b). Correspondingly, the levels of MBP and GST-pi expressions (oligodendrocyte markers) in the magnesium-sulfate-exposed oligodendrocytes were higher compared to vehicle-treated oligodendrocytes (Fig. 2a and c–d). In addition, magnesium-sulfate-treated cells exhibited a higher level of MBP mRNA compared to vehicle-treated OPCs (Supplementary Fig. S1).

In general, mature oligodendrocytes are less vulnerable to ischemic damage compared to OPCs (Volpe et al., 2011). In fact, in our cell culture system, mature cells showed more resistance to OGD stress (Supplementary Fig. S2). Therefore, we examined if magnesium-sulfate-treated cells would be more tolerant of hypoxia/ischemic exposure. After 7 days of magnesium sulfate incubation, differentiated oligodendrocytes were subjected to 2-hr OGD followed by 24-h re-oxygenation (Fig. 3a). Compared to untreated cells, OGD caused a decrease in cell viability in oliogodendrocytes (Fig. 3b). On the other hand, magnesium-sulfate-exposed oligodendrocytes had improved cell viability compared to vehicle-exposed oligodendrocytes after OGD (Fig. 3b). Notably, when

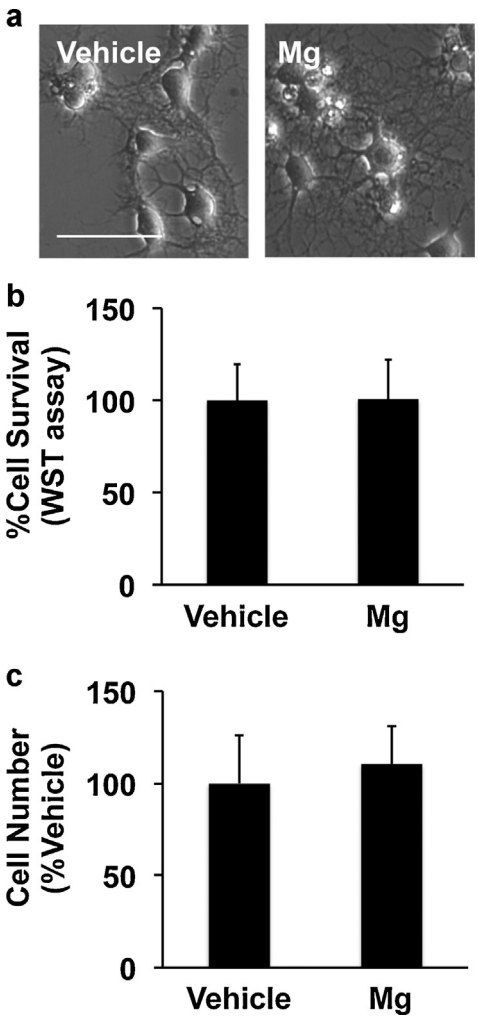

Fig. 1. *Effects of magnesium sulfate on OPC number.* (a) Primary rat OPCs were exposed to vehicle or 5 mM magnesium sulfate (Mg) for 7 days during the time period in which OPCs differentiate into oligodendrocytes. Magnesium-sulfate-treated cells exhibited more maturated oligodendrocytes. Scale bar = 50 μm. (b)–(c) After 7 days of vehicle or magnesium sulfate incubation, cells were subjected to the WST assay or direct cell count. Magnesium sulfate did not affect cell viability of our cultured rat oligodendrocyte lineage cells. Data are represented as Mean ± SD of 3 independent experiments.

magnesium sulfate was added to differentiated oligodendrocytes just before OGD (i.e. during OGD/reoxygenation) or after OGD (i.e. during reoxygenation) (Fig. 4a), magnesium sulfate did not show oligo-protection effects against OGD (Fig. 4b–c), suggesting that magnesium sulfate does not directly protect matuare oligodendrocytes from OGD, but that it supports the progression of OPCs into mature oligodendrocytes, which are more resistant to OGD-induced injury.

Taken together, our current study demonstrates that oligodendrocyte lineage cells with prolonged exposure to magnesium sulfate may acquire resistance to HI stress. Magnesium sulfate may accelerate the differentiation step of oligodendrocyte lineage cells, which contributes to the protection of developing white matter against hypoxic/ischemic stress. Although future deeper studies would be helpful to further dissect the mechanisms of magnesium-sulfate-induced OPC differentiation, our finding is compatible with previous studies that showed that preterm infants who were exposed to magnesium antenatally had a reduced incidence of cerebral palsy (Bozkurt et al., 2015; Rouse et al., 2008). We speculate that infants exposed to magnesium during fetal life, before their preterm delivery, would have an increased number of

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