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Diazoxide Promotes Oligodendrocyte Differentiation in Neonatal Brain in Normoxia and Chronic Sublethal Hypoxia

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Abstract

Periventricular white matter injury (PWMI) is the most common cause of brain injury in preterm infants. It is believed that loss of late oligodendrocyte progenitor cells (OPCs) and disrupted maturation of oligodendrocytes contributes to defective myelination in PWMI. At present, no clinically approved drugs are available for treating PWMI. Previously, we found that diazoxide promotes myelination and attenuates brain injury in the chronic sublethal hypoxia model of PWMI. In this study, we investigated the mechanisms by which diazoxide promotes myelination. We observed that diazoxide increases the ratio of differentiated oligodendrocytes in the cerebral white matter, promotes the expression of differentiation-associated transcriptional factors Nkx2.2 and Sox10, and increases the expression of myelin genes CNP and MBP. These results show that diazoxide promotes oligodendrocyte differentiation in the developing brain.

Keywords

periventricular white matter injury; oligodendrocyte; hypoxia; diazoxide; neonate; mouse

1. Introduction

Periventricular white matter injury (PWMI) is the most common cause of brain injury in preterm infants (Back and Rosenberg, 2014; Salmaso et al., 2014). The period of highest risk for PWMI is 23-32 weeks postconception age, and the risk factors of PWMI include ischemia, hypoxia and inflammation (Borch and Greisen, 1998; Glass et al., 2008; Volpe, 2009). Infants with PWMI are at risk for long-term neurological deficits, including cerebral palsy, cognitive and visual deficits, and learning disabilities (Anderson and Doyle, 2003; Anderson et al., 2011; Johnson et al., 2010). Unfortunately, there are no specific treatments for PWMI at present.

There are two major forms of PWMI that include diffuse white matter injury and focal cystic necrotic lesions (Back and Rivkees, 2004; Back and Rosenberg, 2014; Volpe, 2009). Of

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these types, diffuse white matter injury is the most common form and is characterized by global hypomyelination (Counsell et al., 2003; Hamrick et al., 2004). The period of greatest risk for PWMI is coincident with the onset of oligodendrocyte differentiation and early myelination in the brain when oligodendrocyte progenitor cells (OPCs) and immature oligodendrocytes predominate. It is believed that PWMI involves depletion of late OPCs and disrupted maturation of oligodendrocytes, which leads to impaired myelination (Segovia et al., 2008). Supporting this notion, hypoxia, ischemia, and cytokines induce death of premature oligodendrocytes and cause impaired myelination (Karadottir et al., 2005; Khwaja and Volpe, 2008).

Several lines of evidence suggest that OPCs can regenerate and be recruited to white matter lesions (Aguirre et al., 2007; Frost et al., 2003). However, the differentiation of OPCs into myelinating oligodendrocytes may be impaired (Jablonska et al., 2012; Segovia et al., 2008), contributing to white matter injury (Alix et al., 2012; Chang et al., 2002; Kuhlmann et al., 2009). The ability to stimulate the differentiation of OPCs into myelinating oligodendrocytes is therefore an attractive approach for developing therapies for PMWI. Few agents, however, are known to stimulate oligodendrocyte differentiation *in vivo* (Deshmukh et al., 2013; Fancy et al., 2011; Scafidi et al., 2014).

Recently, we discovered that diazoxide promotes myelination in the neonatal brain and attenuates hypoxia-induced brain injury in neonatal mice (Fogal et al., 2010). Diazoxide is an ATP-sensitive potassium channel activator that is approved by the US Food and Drug Administration (FDA) for the treatment of hyperinsulinism in infants and has a favorable safety profile (Stanley, 2006; Website, 2010). Previous studies demonstrated that diazoxide has neuroprotective effects in ischemia and hypoxia as well (Domoki et al., 1999; Shake et al., 2001). The mechanisms underlying the beneficial effects of diazoxide on myelination, though, remain unknown.

In this study, we examined the effects of diazoxide on oligodendrocyte proliferation and differentiation using the chronic sub-lethal hypoxia (CSH) model of PWMI (Back and Rivkees, 2004; Fogal et al., 2010; Jablonska et al., 2012; Scafidi et al., 2009). We now report that diazoxide promotes the differentiation of oligodendrocytes.

2. Results

2.1. Influence of diazoxide on oligodendrocyte proliferation in cerebral white matter

We first examined the effect of diazoxide on the proliferation rate of OPCs. 5-bromo-2'-deoxyuridine (BrdU) labeling was performed and followed by immunofluorescence staining with anti-BrdU and anti-Olig2 antibodies (Fig. 1). Short-term diazoxide treatment from P3 to P7 did not affect the rates of oligodendrocyte proliferation in the external capsule in normoxic or hypoxic conditions (Fig. 1 E). In addition, the diazoxide-treated mice had a similar density of oligodendrocytes in the external capsule as vehicle-controls (Fig. 1F). These results indicate that diazoxide does not promote proliferation, nor increase the number of oligodendrocytes in the cerebral white matter during neonatal stages.

We also examined the effect of hypoxia on oligodendrocyte proliferation. Short-term hypoxia treatment significantly reduced the proliferation rate of oligodendrocytes in the external capsule by $43.19 \pm 12.83\%$ (Fig. 1E). The density of oligodendrocytes was comparable between normoxia- and hypoxia-reared mice at P7 (Fig. 1F), and we did not detect increased apoptosis in this area (Suppl. Fig. 1). These data indicate that the hypoxia suppresses the proliferation of OPCs in the cerebral white matter.

2.2. Diazoxide influences oligodendrocyte differentiation

We next assessed if diazoxide influences oligodendrocyte differentiation. To calculate the percentage of differentiated oligodendrocytes, we counted the number of APC positive cells and the number of Olig2 positive cells. Under normoxic condition, short-term diazoxide treatment from P3 to P9 significantly increased the proportion of differentiated oligodendrocytes by $24.92 \pm 11.98\%$ as compared with vehicle-controls (Fig. 2E). But, short-term diazoxide did not influence oligodendrocyte differentiation under hypoxic condition (Fig. 2E). Long-term diazoxide treatment from P3 to P12 significantly increased the proportion of differentiated oligodendrocytes by $10.29 \pm 3.78\%$ versus vehicle controls in normoxia, and by $11.05 \pm 3.13\%$ in hypoxia (Fig. 3E). Diazoxide did not change the density of oligodendrocytes at P9 or P12 (Fig. 2F and 3F). These data indicate that the increased proportion of mature cells in oligodendrocytes results from enhanced oligodendrocyte differentiation in the diazoxide-treated animals.

Although long-term hypoxia significantly reduced the oligodendrocyte differentiation by $13.39 \pm 2.66\%$, as compared with normoxic controls (Fig. 3E), short-term hypoxia did not decrease the differentiation level in oligodendrocytes (Fig. 2E). In addition, long-term hypoxia, but not short-term hypoxia, significantly reduced the density of oligodendrocytes in the external capsule (Fig. 2F and 3F). These results indicate that hypoxia does not directly inhibit oligodendrocyte differentiation and the impaired differentiation may result from decreased OPC pool under hypoxic conditions.

2.3. Diazoxide influences oligodendrocyte differentiation-related gene expression

To test if the enhanced oligodendrocyte differentiation was associated with altered transcriptional activity in oligodendrocytes, we examined the expression of the homeobox gene *Nkx2.2* and the SRY-related HMG-box gene *Sox10*, which are transcription factors that promote oligodendrocyte differentiation (Fu et al., 2002; Liu et al., 2007; Qi et al., 2001; Stolt et al., 2002), with quantitative polymerase chain reaction with reverse transcription (qRT-PCR). In normoxia, both short-term and long-term diazoxide treatment significantly increased the expression of *Nkx2.2* and *Sox10* (Fig. 4). Compared with the controls, the expression of *Nkx2.2* was increased by 5.10 ± 0.97 fold at P7 and by 1.73 ± 0.24 fold at P12, and expression of *Sox10* was increased by 3.33 ± 0.79 fold at P7 and by 2.25 ± 0.68 fold at P12. However, diazoxide did not change the expression of *Nkx2.2* and *Sox10* under hypoxic conditions (Fig. 4). These results suggest that diazoxide promotes oligodendrocyte differentiation through stimulating the expression of differentiation-associated transcriptional factors during normal development.

We next examined if the increased expression of Nkx2.2 and Sox10 was associated with altered expression of mature oligodendrocyte markers. For this analysis we quantified the expression of 2', 3'-Cyclic-nucleotide-3'-phosphodiesterase (CNPase) and myelin basic protein (MBP) in forebrain. The qRT-PCR results showed that short term diazoxide treatment significantly increased the expression of CNP (1.48 ± 0.05 fold versus vehicle) and MBP (2.48 ± 0.25 fold versus vehicle) at P7 in normoxia, but not in hypoxia (Fig. 5A, B). Diazoxide treatment from P3 to P12 did not increase the expression of CNP and MBP (Fig. 5C, D). Western blotting showed that long-term diazoxide treatment did not increase the expression of MBP (Supp. Fig. 2). These results indicate that diazoxide promotes differentiation of oligodendrocytes during early neonatal development, which is coincident with increased expression of Nkx2.2 and Sox10 in mice with diazoxide treatment (Fig. 4).

We observed that short-term and long-term hypoxia have different impacts on myelin gene expression. Short-term hypoxia significantly increased the expression of myelin genes, CNP and MBP (Fig. 5A, B). But after long-term hypoxia, the expression of CNP was similar as the normoxic controls (Fig. 5C); and the expression of MBP was significantly reduced compared with the normoxic controls (Fig. 5D). These data indicate that short-term hypoxia induces premature differentiation in oligodendrocytes, while long-term hypoxia results in reduced expression of myelin gene.

3. Discussion

PWMI is associated with loss of OPCs, defective differentiation of oligodendrocytes and impaired myelination. (Back and Rivkees, 2004; Back and Rosenberg, 2014; Salmaso et al., 2014). Therefore, promoting oligodendrocyte differentiation is one potential therapeutic approach for PWMI. We report that diazoxide stimulates the differentiation of oligodendrocytes in normal developing brains and in the model of PWMI.

Under normoxic conditions, we observed that diazoxide promoted oligodendrocyte differentiation, which is consistent with our previous observation that diazoxide improves myelination under normoxic condition (Fogal et al., 2010). Simultaneously, we found that diazoxide significantly increased the expression of oligodendrocyte specific transcriptional factors, Nkx2.2 and Sox10, and the expression of myelin genes, MBP and CNP. Nkx2.2 and Sox10 are oligodendrocyte specific transcriptional factors, which are necessary to promote oligodendrocyte differentiation and able to regulate the transcription of multiple myelin genes (Li et al., 2007; Liu et al., 2007; Qi et al., 2001). Therefore, our observations support the notion that diazoxide promotes oligodendrocyte differentiation by stimulating the expression of Nkx2.2 and Sox10 at neonatal stages (Fig. 6).

In addition, we observed that short-term diazoxide treatment was more potent in promoting differentiation of oligodendrocytes than long-term treatment. The differentiation of oligodendrocytes in the external capsules begins around P7 (Back and Rosenberg, 2014). Thus, most oligodendrocyte lineage cells in this region are immature oligodendrocytes during the short-term treatment period, whereas both immature and mature oligodendrocytes are present during long-term treatment. The observed difference between the short-term and

long-term treatments suggests that effects of diazoxide are most apparent when OPCs and immature oligodendrocytes predominate.

Compared with the normoxic controls, we observed that diazoxide had a modest effect on oligodendrocyte differentiation under hypoxic conditions. We found that long-term diazoxide treatment promoted oligodendrocyte differentiation in the external capsule in hypoxia, which is consistent with our previous finding showing about improved myelination in the external capsule after diazoxide treatment (Fogal et al., 2010). However, results of qPCR and western blotting, which used the whole forebrain to prepare RNA and protein, did not show that diazoxide significantly improves overall expression of myelin gene in the forebrain in hypoxia, suggesting that drug effects may vary by brain region.

In addition, long-term, not short-term, diazoxide treatment promoted oligodendrocyte differentiation, which is probably due to reduced OPC pool under hypoxic conditions, especially in the early phase of hypoxia. We found that short-term hypoxia treatment resulted in reduced proliferation and premature differentiation in oligodendrocytes, which might reduce the OPC pool. In this circumstance, diazoxide showed little impact on oligodendrocyte differentiation. However, previous studies reported that oligodendrocyte proliferation in cerebral white matter is higher than normoxic controls after long-term hypoxia treatment (Chew et al., 2010). Enhanced proliferation can result in a larger OPC pool, which is required for diazoxide action. Therefore, the observed effects of diazoxide in hypoxia support the notion that diazoxide action is dependent on the presence of OPCs and immature oligodendrocytes.

Although our previous studies showed that diazoxide promotes proliferation of OPCs *in vitro* (Fogal et al., 2010), we did not detect change in proliferation of OPCs in the white matter after diazoxide treatment. The oligodendrocytes examined in this study were late OPCs. This finding suggests that diazoxide does not stimulate proliferation in late OPCs in the cerebral white matter.

Overall, we find that diazoxide can stimulate oligodendrocyte differentiation, and the drug effects are dependent on developmental stage of oligodendrocyte lineage cells. These observations support the notion that diazoxide has potential to stimulate myelination in PWMI.

4. Materials and methods

4.1. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida, College of Medicine. C57/BL6 mice were obtained from Charles River Laboratories (Wilmington, MA). The rodent chronic sublethal hypoxia model was used as reported (Fogal et al., 2010). Briefly, postnatal day 3 (P3) C57/BL6 pups and their dams were placed in a Plexiglas hypoxia chamber along with cross-fostered CD-1 dams. The oxygen concentration was maintained at $10\pm 1\%$. Pups reared in normoxia and hypoxia received subcutaneous injections of vehicle or diazoxide (10mg/kg) daily. The "short term" treatment periods were from P3 to P7 or P3 to P9. The "long term" treatment

period was from P3 to P12. The stock solution of diazoxide (20 mg/ml in dimethyl sulfoxide) was diluted in phosphate buffered saline (PBS, pH=7.4) immediately before injection.

4.2. Immunofluorescence staining

Mouse pups were perfused with PBS and 4% paraformaldehyde (PFA) before tissue collection. Brain tissue was post-fixed in 4% PFA overnight at 4 °C and stored in PBS with 0.02% sodium azide at 4 °C. Free floating thick sections (60 µm) were cut in a Vibratome (Pelco 10190, Redding, CA). Sections from the start of corpus callosum to the rostral end of the third ventricle were collected and used for immunofluorescence staining. For proliferation assays, BrdU labeling and immunostaining were performed as described (Zhu et al., 2010). Alexa488 conjugated-anti-BrdU antibody (Invitrogen) was used to label BrdU-positive cells. For differentiation assays, anti-Olig2 (Millipore) and anti-APC (Calbiochem) antibodies were used. The second antibodies were Alexa Fluor® 594 Goat Anti-Rabbit Antibody (Molecular Probe) and Alexa Fluor® 488 Goat Anti-Mouse IgG2b Antibody (Molecular Probe). For apoptosis assays, immunostaining was performed with anti-activated-Caspase3 (pCas3, BD), anti-Olig2 (Millipore), and anti-APC antibodies. The second antibodies were Alexa Fluor® 647 Goat Anti-rabbit Antibody (Molecular Probe), Alexa Fluor® 568 Goat Anti-Mouse IgG2a Antibody (Molecular Probe), and Alexa Fluor® 488 Goat Anti-mouse IgG2b Antibody (Molecular Probe).

An Olympus IX81 spinning disk confocal optical microscope was used to capture images of immunostained brain sections (20-30 µm/section). Four different lasers were used to image FITC, Cy5, TexRed and DAPI, respectively. The stacks were z axis (9 µm) collapsed to analyze cellular morphology and identify fluorescent labels of specific cell types. We analyzed images from four consecutive sections for each animal. At least 8 images per animal, and at least 3 animals per group, were analyzed. Quantification was limited to the external capsules, the boundaries of which were defined by the distribution of 40, 6-diamidino-2-phenylindole (DAPI) and Olig2 signals. Total and relative numbers of cells were analyzed by the number of cells labeled with specific markers. Olig2 labels oligodendrocyte lineage cells; APC labels mature oligodendrocytes; BrdU labels dividing cells; and pCas3 labels apoptotic cells. We counted the number of Olig2+ cells with the plugin of Analyze Particle (Image J 1.46R) and the number of double-labeled cells manually.

4.3. qRT-PCR

Whole brains were dissected on ice. Forebrain tissue was obtained by removing olfactory bulbs, cerebellums and midbrain with fine scissors along the anterior and posterior edges of cerebrum. Total RNA was prepared from forebrain tissue with RNeasy Plus (Qiagen) and treated with DNase I (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from total RNA with BioRad 5× iScript kit (Bio-Rad). qPCR was performed with SYBR Green PCR master Mix (Applied Biosystems) with Applied Biosystems 7300 Real Time PCR System (Applied Biosystems). The qPCR conditions were: step 1, 95 °C for 5 min; step 2, 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, for 40 cycles. The sequences of qPCR primers used in this study are listed in Supplemental Table 1.

4.4. Western Blot Analysis

Forebrain tissues, which were dissected from whole brains as described above, were lysed and homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer (Thermo Scientific) supplemented with Complete Mini Proteinase Inhibitor Cocktail Tablets (Roche) using a microtube homogenizer (Benchmark Scientific). Protein samples (50 ug/lane) were separated on 15% Criterion Tris-HCl Precast Gel (BioRad), and Western blotting was performed, as described (Zhu et al., 2013). The target proteins were probed by anti-MBP (Covance) and anti-*p*-actin (Sigma) antibodies. *p*-actin was used as the loading control. Images on blots were detected with Pierce ECL-2 Western blotting substrate (Pierce) in ChemiDoc XRS+ Imaging System (Bio-Rad). Band densitometry was quantitated using BioRad Image Lab 4.1 (Bio-Rad).

4.5. Statistics

Results were analyzed with GraphPad Prism 6.02 (GraphPad Software). Data were presented as mean $\pm$ SEM. Statistical differences between groups were determined using two-way ANOVA followed by uncorrected Fisher's test. Individual *p* values were given for each comparison. *P*<0.05 was considered to be statistically significant. For qPCR analysis, linearized 2^{-CT} values were used to determine statistical differences between groups and the 2^{-CT} method was used to determine the fold change between the treatment groups and controls (Fang et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Diazoxide increases oligodendrocyte differentiation in the cerebral white matter.
- Diazoxide promotes the expression of transcriptional factors, Nkx2.2 and Sox10 in normoxia.
- Diazoxide increases the expression of myelin genes, CNP and MBP, in normoxia.

Figure 1. Effects of diazoxide and hypoxia on oligodendrocyte proliferation in the external capsule

(A-D) Confocal images of P7 cerebral sections from normoxia and hypoxia reared mice with treatment of vehicle or diazoxide from P3 to P7. Tissue sections were stained with anti-Olig2 (red) and anti-BrdU (green) antibodies and nuclei were counterstained with DAPI (blue). Dashed lines mark the external capsule. Arrows indicate anti-Olig2 and anti-BrdU double-labeled proliferating oligodendrocytes; arrow heads indicate Olig2+ cells. Scale bar represents 50 μ m. (E) Quantification of percentage of Olig2+ cells that are co-labeled with BrdU in the external capsule. OL, oligodendrocyte. (F) Quantification of the density of oligodendrocytes in the external capsule. $n_{\text{normoxia}}=3$, $n_{\text{hypoxia}}=3$. Two way ANOVA with uncorrected Fisher's LSD, **, $p<0.01$. Error bars represent standard error mean (SEM).

Figure 2. Effects of diazoxide and hypoxia on differentiation in oligodendrocytes at P9 (A-D). Confocal images of P9 cerebral sections from normoxia and hypoxia reared mice with treatment of vehicle or diazoxide from P3 to P9. Sections were double-labeled with anti-Olig2 (red) and anti-APC (green) antibodies and nuclei were counterstained with DAPI (blue). Arrows indicate anti-Olig2 and anti-APC co-labeled mature oligodendrocytes and arrow heads indicate Olig2+ cells. Scale bar represents 50 μm . (E) Quantification of differentiated cells in Olig2+ cells in the external capsule. OL, oligodendrocyte. (F) Quantification of the density of oligodendrocytes in the external capsule. $n_{\text{normoxia}}=3$,

$n_{\text{hypoxia}}=4$. Two way ANOVA with uncorrected Fisher's LSD, **, $p < 0.01$. Error bars represent SEM.

Figure 3. Effects of diazoxide and hypoxia on differentiation in oligodendrocytes at P12 (A-D). Confocal images of P12 cerebral sections from normoxia and hypoxia reared mice with treatment of vehicle or diazoxide from P3 to P12. Sections were double-labeled with anti-Olig2 (red) and anti-APC (green) antibodies and nuclei were counterstained with DAPI (blue). Arrows indicate anti-Olig2 and anti-APC co-labeled mature oligodendrocytes and arrow heads indicate Olig2+ cells. Scale bar represents 50 μ m. (E) Quantification of differentiated cells in Olig2+ cells in the external capsule. OL, oligodendrocyte. (F) Quantification of the density of oligodendrocytes in the external capsule. $n_{\text{normoxia}}=3$,

$n_{\text{hypoxia}}=6$. Two way ANOVA with uncorrected Fisher's LSD, *, $p<0.05$. Error bars represent SEM.

Figure 4. Diazoxide increases the expression of Nkx2.2 and Sox10

(A-D) Quantification of the mRNA expression of Nkx2.2 and Sox10 at P7 and P12 with qRT-PCR. Bars represent the fold change relative to normoxia vehicle control. $n_{\text{normoxia-P7}}=3$, $n_{\text{hypoxia-P7}}=4$, $n_{\text{normoxia-P12}}=6$, $n_{\text{hypoxia-P12}}=6$. Two way ANOVA with uncorrected Fisher's LSD, * $p < 0.05$, ** $p < 0.01$. Error bars represent SEM.

Figure 5. Diazoxide and hypoxia influence the expression of CNP and MBP

(A-D) Quantification of the mRNA expression of CNP and MBP at P7 and P12 with qRT-PCR. Bars represent the fold change relative to normoxia vehicle control. $n_{\text{normoxia-P7}}=3$, $n_{\text{hypoxia-P7}}=4$, $n_{\text{normoxia-P12}}=6$, $n_{\text{hypoxia-P12}}=6$. Two way ANOVA with uncorrected Fisher's LSD, * $p<0.05$, ** $p<0.01$. Error bars represent SEM.

Figure 6. Cartoon depicting effects of diazoxide on oligodendrocyte differentiation during neonatal development

In normoxia, diazoxide opens ATP-sensitive potassium channel, which results in increased expression of Nkx2.2 and Sox10. Consequently, the increased activities of Nkx2.2 and Sox10 promote oligodendrocyte differentiation and the expression of CNP and MBP.