Journal Section: Neurobiology of Disease

Glucocorticoid Protection of Oligodendrocytes against Excitotoxin Involving Hypoxia-Inducible Factor-1α in a Cell Type-Specific Manner

Abbreviated title: GC Protection of OLGs Involves HIF-1α/Epo

Yu-Yo Sun[1,2], Chen-Yu Wang[1], Ming-Feng Hsu[3,4], Shu-Hui Juan[1,2], Chiu-Yun Chang[5], Chih-Ming Chou[6], Liang-Yo Yang[1,2], Kuo-Sheng Hung[7], Jan Xu[8], Yi-Hsuan Lee[1,2,10] & Chung Y. Hsu[2,9,10]

[1]Department of Physiology, [2]Division of Cell Physiology and Neuroscience, Graduate Institute of Medical Sciences, [5]Department of Anatomy, [6]Department of Biochemistry, [7]Institute of Injury Prevention, Taipei Medical University, Taipei 110, Taiwan; [3]Institute of Biological Chemistry, [4]Core Facility for Protein Production and X-ray Structural Analysis, Academia Sinica, Taipei 115, Taiwan; [8]Department of Neurology, Washington University Medical Center, St. Louis, MO 63110, USA; [9]Graduate Institute of Clinical Medical Sciences, China Medical University, Taichung 404, Taiwan

[10]Corresponding authors:

Yi-Hsuan Lee, Ph.D., Department of Physiology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, Tel: +886-2-27361661 ext. 3188; Fax: +886-2-23781073; E-mail: hsuan@tmu.edu.tw

Chung Y. Hsu, M.D., Ph.D., Graduate Institute of Clinical Medical Sciences, China

Medical University, Taichung, Taiwan. E-mail: hsuc@mail.cmu.edu.tw

Number of figures and tables: 7 figures

Contents of supplemental material: 2 supplementary figures

Number of pages: 35 pages

Number of words for Abstract (198 words), Introduction (499 words), and Discussion (876 words)

Keywords

Oligodendrocytes; glucocorticoid receptor; methylprednisolone; HIF-1α; erythropoietin; white matter injuries

Acknowledgments

We thank C. Y. Kuan, J. Ma, and H. T. Chang for critiques. This work was supported by National Research Genomic Medicine Project 93GMP002-1, 93GMP002-2, 93GMP002-5, and NSC96-2628-B-038-010-MY3 of National Science Council in Taiwan.

Glucocorticoids are commonly used in treating diseases with white matter lesions including demyelinating diseases and spinal cord injury (SCI). However, glucocorticoids are ineffective in gray matter injuries, such as head injury and stroke. The differential glucocorticoid effects in white and gray matter injuries are unclear. We report here a novel mechanism of methylprednisolone (MP), a synthetic glucocorticoid widely used for treating multiple sclerosis and SCI, in protecting oligodendrocytes (OLGs) against α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-induced excitotoxicity, which has been implicated in the white matter injuries and diseases. The cytoprotective action of MP in OLGs is causally related to its upregulation of a neuroprotective cytokine erythropoietin (Epo). MP transactivation of *Epo* expression involves dual transcription factors, glucocorticoid receptor (GR) and hypoxia-inducible factor-1α (HIF-1α). Co-immunoprecipitation, chromatin immunoprecipitation analysis, yeast two-hybrid analysis, and structure modeling of three-dimensional protein-protein interactions confirm MP-induced interaction between GR DNA binding domain and HIF-1α PAS domain in the transactivation of *Epo* in OLGs. In contrast, MP activates GR, but does not induce GR-HIF-1α interaction, HIF-1α binding to *Epo* enhancer/promoter, or Epo expression in cultured cortical neurons. The OLG-specific GR-HIF-1α transactivation of *Epo* provides novel insights into the development of more effective therapies for diseases affecting the white matter.

Introduction

Neuroprotective strategies for treating central nervous system (CNS) disorders have been directed mainly at preserving gray matter or neurons (Lipton, 2006). However, white matter injuries, as seen in demyelinating diseases including multiple sclerosis (MS) and spinal cord injury (SCI) may have an even greater impact on functional outcomes (Bradbury et al., 2002; Stys and Lipton, 2007). For example, disability after SCI is primarily caused by the white matter lesion (Wrathall et al., 1994; Crowe et al., 1997). Gray matter injury, sparing most white matter (e.g., central cord syndrome) results in segmental motor/sensory dysfunction. Disability after SCI can be largely ascribed to injury to the white matter which contains the long tracts. Death of OLGs, that form myelin sheath and maintain long tract function in the CNS, is a prominent pathological feature in demyelinating diseases and SCI (Crowe et al., 1997; Miller and Mi, 2007). OLG death in these disorders results, in part, from excitotoxicity involving AMPA receptor (McDonald et al., 1998; Smith et al., 2000; Miller and Mi, 2007). AMPA receptor antagonism is effective in salvaging white matter after traumatic or ischemic SCI (Wrathall et al., 1994; Kanellopoulos et al., 2000).

刪除: mostly

Glucocorticoids (GCs), primarily methylprednisolone (MP), are the mainstay in treating patients with diseases affecting the white matter, including MS and SCI (Bracken et al., 1990; Zivadinov et al., 2001; Chataway et al., 2006). GCs regulate transcriptional events by activating glucocorticoid receptor (GR), which binds to glucocorticoid response

element (GRE) or interacts with other transcription factors to regulate gene expression (Becker et al., 1986). GR modulation of inflammatory/immune or oxidative processes are the proposed mechanisms of MP action in demyelinating diseases and SCI (Schmidt et al., 2000; Xu et al., 2001a; Tsutsui et al., 2008). However, GC or antioxidant therapies were ineffective in gray matter injuries such as head trauma or stroke (Phil et al., 2005; Diener et al., 2008). The mechanism underlying preferential effects of GCs in the white, but not gray, matter remains to be defined.

MP upregulates anti-apoptotic *Bcl-x_L* gene expression to protect OLGs against AMPA-induced death in vitro and in a rat SCI model in vivo (Lee et al., 2008). Bcl-x_L expression is also upregulated by erythropoietin (Epo)/Epo receptor (Silva et al., 1999). Epo is effective in animal models of SCI and MS (Gorio et al., 2002; Li et al., 2004). Epo upregulation involves activation of hypoxia-inducible factor-1α (HIF-1α) (Semenza et al., 1991; Liu et al., 2005), a member of the PAS (PER/ARNT/SIM) family of basic helix-loop-helix (bHLH) transcription factors essential in hypoxia sensing for cell survival (Sharp and Bernaudin, 2004; Semenza, 2009). Upon activation, HIF-1α translocates into the nucleus, heterodimerizes with ARNT to bind to hypoxia-responsive elements (HRE) in the 3' enhancer of the *Epo* gene to induce *Epo* expression (Semenza et al., 1991).

We report here *Epo* transactivation involving GR and HIF-1α interaction is a causal mechanism of MP protection of OLGs, but not neurons, against AMPA excitotoxicity.

These findings offer novel insights into the development of innovative therapies for white matter diseases.

Materials and Methods

Animals

Sprague Dawley (SD) rats were obtained from the National Institute of Experimental Animal Research, Taipei, Taiwan. Animals were sacrificed by an overdose of sevoflurane to minimize pain or discomfort. All animal experimentation procedures were approved by the Experimental Animal Review Committee at Taipei Medical University and are in accordance with the Guide for the Care and Use of Laboratory Animals, the National Institute of Health (USA) guidelines.

Cell cultures

OLGs in culture were prepared from postnatal day 0-2 neonatal rat brains (Xu et al., 2001b; Lee et al., 2004). OLG purity was more than 90 % based on Rip immunoreactivity (Xu et al., 2001b; Lee et al., 2004). Cortical neurons were prepared from fetal rat brains at embryonic day 17 as described previously with approximately 85% cells in culture expressing neuronal marker MAP-2 (Lee et al., 2008). Cortical neurons at 10 days *in vitro* (DIV) that express the glucocorticoid receptor were used in this study.

Quantitative real-time and semi-quantitative RT-PCR

Total RNA extraction, cDNA preparation, and RT-PCR were as described previously (Lee et al., 2008). The real-time PCR reaction was performed using an ABI PRISM 7300 Sequence Detection System. The semi-quantitative PCR of *Epo, Nr3c1* (GR), *Hif1a* (HIF-1α)*, and* *Bcl-x*$_L$ cDNA was performed using the following primers with cDNA of a reference gene *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) measured in parallel as an internal control: *Epo*, 5'-TGCGACAGTCGCGTTCTGGAGAGGTAC-3′ and 5'-ATCCGCTGTGAGTGTTCGGAGTGGAGC-3′; *Nr3c1* (GR), 5'-AGAATGTCTCTACCCTGCATGTATGAC-3′ and 5'-CTGGAAGCAGTAGGTAAGGAGATTCTC-3′; *Hif1a* (HIF-1α), 5'-CAAGATCAGCCAGCAAGTCCTTCTGATG-3′ and 5'-AGGTTTCTGTAACTGGGTCTGCTGGAATC-3′; *Bcl-x*$_L$, 5'-AGGCTGGCGATGAGTTTGAA-3′ and 5'-TGAAACGCTCCTGGCCTTTC-3′; and *Gapdh*, 5'- GACCCCTTCATTGACCTCAAC-3′ and 5'-GATGACCTTGCCCACAGCCTT-3′.

删除: s

删除: and

删除: was

SCI animal model

SCI was conducted using a New York University (NYU) Impactor (Hashimoto et al., 2007)

to injure rat spinal cord at T9-T10 (Lee et al., 2008). SD rats (9-week old) were randomly divided into 3 groups: the sham control, SCI, and SCI with MP treatment groups. Vehicle or MP (30 mg/kg body weight) was administered intravenously 30 min after SCI. Four and 24 h after treatment, animals were anesthetized and a 1-cm segment of the injured cord centering at the injury epicenter or corresponding sham-operated cord tissue was collected for quantitative real-time RT-PCR analysis.

Western blot analysis

Western blot analysis was performed as described previously (Xu et al., 2001b; Lee et al., 2008). Primary antibodies used were rabbit anti-Bcl-x_L (1:500; Santa Cruz), rabbit anti-GR (1:500; Santa Cruz), mouse anti-HIF-1α (1:500; Novus), mouse anti-ARNT (1:1000; Chemicon) and mouse anti-GAPDH (1:5,000; Biogenesis) antibodies. The immune complex was visualized using HRP-reactive ECL reagents (Amersham).

RNA knockdown

OLGs were transfected with a set of siRNAs specific for GR, HIF-1α, Epo, respectively or scrambled RNA (Silencer Pre-designed siRNA, Ambion) using Lipofectamine^{TM} reagent (Invitrogen). The siRNA sequences for GR (Nr3c1; Accession No. NM_012576) were 5’-GGUUGCUUAAAGAAAGUUtt -’3 (exon 9) and 5’- GCUACAGUCAAGGUUUCUGtt -

’3 (exon 1); for HIF-1α (Hif1a; Accession No. NM_024359) 5’-GCUUGCUCAUCAGUUGCCAtt -’3 (exon 2) and 5’- CCAGUUGAAUCUUCAGAUtt -’3 (exon 9) and for Epo (Accession No. NM_017001) 5’-GGAGGCAGAAAAUGUCACAtt -’3 (exon 2) and 5’-GGCUGUAGAAGUUUGGCAAtt -’3 (exon 4). Five hours after transfection, the Lipofectamine-containing medium was replaced with OLG culture medium to allow cells to recover for another 67 h.

刪除: were

刪除:);

刪除: were

Luciferase activity assay

Three copies of human *EPO* gene enhancer (HRE$_{EPO}$)-containing sequences, 5’-ATAGGTACCGCCCTACGTGCTGTCTCAGCCCTACGTGCTGTCTCAGCCCTACGTGCTGTCTCAGCTAGCTAT-3’, were cloned into promoter of pGL2 promoter vector (Promega) to obtain the luciferase reporter construct pHRE$_{EPO}$-Luc. The cells were transfected with pHRE$_{EPO}$-Luc and pRL-TK (Promega) in Lipofectamine^{TM} for 36 h, followed by treatments with various agents for 24 h, and then harvested for the luciferase activity assay using the Dual-Luciferase$^{®}$ reporter assay system (Promega).

刪除:

Co-immunoprecipitation

Nuclear fraction of OLGs was extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce). Nuclear proteins (50 μg) were incubated with mouse anti-GR

antibody (2 μg; Abcam) for 12 h at 4°C, followed with protein G Sepharose for 1 h at 25°C. The immune complex was then washed and dissociated according to the Protein G Sepharose 4 Fast Flow protocol (Amersham). Dissociated immune complex was then subjected to Western blotting using rabbit anti-GR (1:500; Santa Cruz) and mouse anti-HIF-1α (1:500; Novus) antibody, respectively.

Yeast two-hybrid assay

Yeast two-hybrid assay was performed as described previously with minor modifications (Wang et al., 2004). The plasmid pGBKT7-mHIF1α-PAS-B was constructed by cloning a PAS domain (a.a. 238- 346) of HIF1α into pGBKT7 (Clontech). pGADT7-GR-DBD (a.a. 436-516) and pGADT7-GR-LBD (a.a. 601-751) were cloned by inserting DNA fragments of GR derived from pcDNA3.1-rGR (kindly provided by Dr. Trevor K. Archer, LMC, NIEHS, NIH, Research Triangle Park, NC) between the *NdeI* and *XhoI* sites of pGADT7 (Clontech). Y190 cells were co-transformed with GAL4AD-GR-DBD (pGADT7-GR-DBD) and GAL4BD-HIF-1α PAS-A (pGBKT7-HIF-1α PAS-A), or with GAL4AD-GR-LBD (pGADT7-GR-LBD) and GAL4BD-HIF-1α PAS-B (pGBKT7-HIF-1α PAS-B) based plasmids. The transformed cells were plated onto a 25 mM 3-AT containing SD medium (Synthetic Dropout) lacking tryptophan, histidine and leucine. Five of independent colonies were inoculated to examine β-galactosidase activity using *o*-nitrophenyl-*β*-D-

刪除: pcDNA3.1-rGR

刪除:

刪除:

刪除:

galactopyranoside (ONPG) as the substrate with A_{420nm} measured in the liquid culture assay. The β-galactosidase unit was calculated by the equation: β-galactosidase units = 1000 x $A_{420}/(t \times V \times A_{600})$, where t is elapsed time (min) of incubation, V is 0.1 ml x concentration factor, and A_{600} the density of yeast culture.

Chromatin immunoprecipitation (ChIP) assay (Lin et al., 2008)

Antibodies used for immunoprecipitation were mouse anti-GR (2 μg; Abcam), mouse anti-HIF-1α (2 μg; Novus) and rabbit anti-CBP (2 μg; Santa Cruz) antibody. Immune complex-bound DNA was purified using a PCR purification kit (Qiagen) and amplified by quantitative PCR (Applied Biosystems) with primers flanking a HRE-containing *Epo* gene 3' enhancer fragment (+3497 ~ +3618, NM-017001), 5'-TACCTCCCCCCCCCCCATTCTGGT-3' and 5'-CAAGCCCAGAGGGGTCAAGAGGTCAGA-3'. Primers flanking an *Epo* gene promoter fragment (-375 ~ -221, NM-017001) were 5'-CAGCCTGCTCTACCCCAGCAAGGA-3' and 5'-GGGGGTCGGGGATGTTATCAGCA-3'. Primers flanking a GRE-containing *Trh* gene promoter fragment (-267 ~ -71, NM-013046) were 5'-CGCGGACTACTCCCGGGACGTCTCT-3' and 5'-GGGGAGGGGGCGCAGGCCGAAGACA-3'. Normal mouse IgG served as the control.

Computer modeling of protein-protein interaction

The structures of GR and HIF-1α were obtained as described below for the predictive structural analysis of GR-HIF-1α interaction. For GR, the structure of GR-DBD (a.a. 439-510) is available (PDB No. 1GDC), and GR-LBD (a.a. 519-795) was generated by SWISS-MODEL Repository (Schwede et al., 2003) using the protein template PDB No. 1 NHZ, 1M2Z and 1P93. No crystal structure information of HIF-1α is available. We used conserved domain search of NCBI BLASTp for sequence domain analysis. The PAS-B domain of HIF-1α was predicted by sequence homology (PDB No. 1 P97, A24A, A24B, 1XO0 and 1WA9). Furthermore, Fold Recognition and 3D-Annotation were conducted to obtain a predicted 3D structure. Prediction of interactions of the GR-DBD and GR-LBD with the PAS-B domain of the HIF-1α was then carried out using program GRAMM (Vakser, 1996). In addition, we predicted the interaction of HIF-1α and ARNT using the crystal structures of HIF-2α-ARNT PAS-B domain complex (PDB No. 2A24) as a template, followed by predicting the interaction of GR-DBD and GR-LBD with the HIF-1α-ANRT complex. Finally, the DNA binding capability of GR-DBD in docking into the HIF-1α or HIF-1α-ARNT complex was predicted.

Statistic analysis

Data were expressed as means ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls multiple comparison posttest to compare all groups with the control group or to compare designated pairs of groups. A p value < 0.05 was considered significant.

Results

MP upregulation of *Epo* expression in OLGs but not cortical neurons.

We noted in a recent study that neuroprotective effect of MP is restricted to OLGs, but not neurons, in the injured spinal cord (Lee et al., 2008). MP or other GCs are effective in treating white (Zivadinov et al., 2001; Chataway et al., 2006), but not gray (Phil et al., 2005; Diener et al., 2008) matter diseases. Epo has similar effects in SCI (Gorio et al., 2002; Vitellaro-Zuccarello et al., 2007) and the white matter diseases (Li et al., 2004). These findings suggest MP and Epo may share a common neuroprotective mechanism. Fig. 1*A* shows that MP upregulated *Epo* mRNA expression in OLGs, but not neurons, based on semi-quantitative (upper panel) and quantitative (lower panel) RT-PCR analysis. *Epo* transactivation has been shown to involve HIF-1α, a cytoprotective transcription factor, inducible by hypoxia (Semenza et al., 1991; Sharp and Bernaudin, 2004; Semenza, 2009). Cobalt chloride ($CoCl_2$) activates HIF-1α, mimicking hypoxia. While MP failed to upregulate *Epo* expression in neurons, chemical hypoxia induced by $CoCl_2$ was effective in

neurons as well as OLGs (Fig. 1*A*, upper panel). This finding suggests that neuronal cultures used in the present study maintain a hypoxia-inducible response for upregulating *Epo* expression, but do not share MP responsiveness noted in OLGs. The ELISA results show that MP increased the Epo protein content in OLGs in a dose-dependent manner with an EC 50 of 83.11 nM (Fig. 1*B*).

MP upregulation of *Epo* expression in the injured spinal cord *in vivo*.

We further examined whether MP treatment affects *Epo* gene expression in the injured spinal cord in rats. Fig. 1*C* shows MP upregulation of *Epo* mRNA expression in the spinal cord 4 and 24 h after injury with a dosing schedule (intravenous MP, 30 mg/kg body weight given 30 min after insult) that has been shown to reduce the extent of OLG apoptosis and white matter damage after SCI (Lee et al., 2008). Results suggest that MP-induced *Epo* expression occurs not only in cultured OLGs *in vitro* but also in the injured spinal cord *in vivo*.

Causal role of *Epo* expression in MP protection of OLGs against excitotoxicity.

To establish the causal role of *Epo* expression in MP protection of OLGs against excitotoxicity, we first studied whether exogenous Epo shares a similar cytoprotective action as MP in OLGs. Both EPO (Silva et al., 1999) and MP (Xu et al., 2009) engage the

JAK/STAT5 pathway in upregulating *Bcl-x_L*, an anti-apoptotic gene. Both human recombinant EPO (rhEPO, 40 ng/ml) and MP upregulated *Bcl-x_L* expression in AMPA-treated OLGs (Fig. 2*A*), sharing a common cytoprotective mechanism. Application of rhEPO also protected OLGs against AMPA-induced death (Fig. 2*B*). To confirm the causal role of Epo expression in MP protection of OLGs, we applied *Epo* siRNA (siEpo) to knock down *Epo* expression (Fig. 2*C*, inset). The cytoprotective action of MP was abolished in OLGs transfected with siEpo, but not scrambled RNA (Fig. 2*C*). These findings support the contention that *Epo* expression is causally related to MP protection of OLGs against AMPA excitotoxicity.

GR-dependent activation of HIF-1α by MP.

Transactivation of *Epo* gene entails the activation of HIF-1α to bind to HRE in the 3' *Epo* enhancer region (Semenza et al., 1991). Therefore, we examined whether MP upregulation of *Epo* expression is mediated by HIF-1α activation. We established a luciferase reporter construct by inserting a HRE-containing DNA fragment of the *Epo* gene enhancer into a pGL2-promoter vector (pHRE$_{EPO}$-Luc) to study the effect of MP on *Epo* expression in relation to HIF-1α activity. Treatment with 1 μM MP increased HRE$_{EPO}$-driven luciferase activity in OLGs, but not neurons (Fig. 3*A*). The MP effect was comparable to a positive control entailing chemical hypoxia with $CoCl_2$ exposure (Fig. 3*B*), confirming that this

expression construct is responsive to an established HIF-1α activation mechanism. Fig. 3*C* shows that dexamethasone, a potent GR agonist, increased HRE_{EPO}-driven luciferase activity, comparable to MP. Non-GC steroids, such as aldosterone, a mineralocorticoid receptor (MR) agonist, or 17β-estradiol, an estrogen receptor agonist, were without effects. GR-dependent action of MP was further confirmed by the findings that RU486 (a GR antagonist), but not spironolactone (an MR antagonist), was effective in blocking MP activation of HIF-1α at the basal level (Fig. 3*D*) or after AMPA treatment (Fig. 3*E*).

Reversal of MP activation of $pHRE_{EPO}$-Luc and upregulation of *Epo* expression by GR or HIF-1α knockdown.

To confirm the causal role of GR and HIF-1α in *Epo* expression and subsequent Bcl-x_L upregulation by MP, we transfected OLGs with specific siRNAs against *Nr3c1* (siGR) or *Hif1a* (siHif1a) to knockdown GR and HIF-1α expression, respectively. Both mRNA and protein levels of GR and HIF-1α were reduced by their specific siRNA (Supplementary Fig. 1*A*, 1*B*). To verify that siHif1a indeed reduces HIF-1α at the protein level, we used a strategy reported previously (Siddiq et al., 2009) to confirm the knockdown efficiency of siHIF-1α in hypoxia-treated OLGs (Supplementary Fig. 1*C*). GR or Hif1a knockdown abolished MP activation of $pHRE_{EPO}$-Luc (Fig. 4*A*), MP upregulation of *Epo* (Fig. 4*B*) and *Bcl-x_L* (Fig. 4*C*), and the protective effect of MP against AMPA-induced cell death in OLGs

(Fig. 4*D*). HIF-1α knockdown even accentuated AMPA excitotoxicity (Fig. 4*D*). These results support the causal role of GR and HIF-1α in mediating the cytoprotective action of MP via upregulation of *Epo* expression in OLGs.

MP enhancement of nuclear GR-HIF-1α interaction in OLGs.

GR may regulate gene expression by interacting with other transcription factors (Xu et al., 2009). We explored whether GR interacts with HIF-1α directly in MP transactivation of *Epo* gene expression. The blotting pattern of HIF-1α, verified by $CoCl_2$ (Supplementary Fig. 1*C*), was used to identify HIF-1α in the co-immunoprecipitation (co-IP) study. MP treatment increased nuclear HIF-1α accumulation in OLGs, but not neurons (Fig. 5*A*). The GR content in the nuclear fraction was increased in OLGs as well as neurons following MP treatment (Fig. 5*B*). However, an increase in GR-HIF-1α interaction following MP treatment was noted only in OLGs, but not neurons (Fig. 5*B*); in agreement with earlier findings that MP transactivates *Epo* expression only in OLGs, but not neurons.

Co-immunoprecipitation of two proteins can result from either direct or indirect interaction. We conducted yeast two-hybrid analysis to explore possible direct interaction between GR and HIF-1α. Based on the domain maps of GR and HIF-1α (Fig. 5*C*), we examined the interactions between the GR ligand binding domain (GR-LBD) and DNA binding domain (GR-DBD) respectively with the HIF-1α PAS-A (a.a. 85-153) and PAS-B

domains (a.a. 238-346). The GR-DBD, but not the GR-LBD, yielded appreciable number of colonies when hybridized with the HIF-1α PAS-A or PAS-B domain (Fig. 5*D*). Results suggest that the GR-DBD, but not the GR-LBD, directly interacts with the HIF-1α PAS domains.

Co-recruitment of HIF-1α and GR onto the promoter and 3' enhancer of the *Epo* gene in OLGs but not neurons.

Since MP induces GR-HIF-1α interaction (Fig. 5), we examined whether the GR-HIF-1α complex is recruited to the promoter or/and 3' enhancer of the *Epo* gene upon MP treatment. Chromatin immunoprecipitation (ChIP) analysis was performed by immunoprecipitating nuclear HIF-1α or GR, followed by PCR amplification of promoter fragments spanning from -375 to -221 and HRE-containing 3' enhancer fragments spanning from +3497 to +3618 of the *Epo* gene, respectively (Fig. 6*A*). Preferential effects of MP on the GR/HIF-1α association with the *Epo* promoter and enhancer were observed in OLGs (Fig. 6*B*) in comparison with neurons (Fig. 6*C*). In OLGs but not neurons, the association of HIF-1α and GR to the *Epo* promoter was increased to 3 folds at 1 h and 3 h after MP treatment (Fig. 6*B*). Interestingly, GR and HIF-1α binding to the 3' enhancer was not significant until 3 h after MP treatment when approximately 2.5-fold and 7-fold increase was noticed in comparison with basal binding activity (0 h), respectively. In neurons, HIF-

1α had a slight and transient association with the *Epo* enhancer after MP treatment. No significant association of HIF-1α with the promoter was observed in neurons. More importantly, no significant GR binding to either the promoter or 3' enhancer was noted in neurons (Fig. 6*C*). As a positive control, MP-induced GR binding to a GRE-containing fragment in the promoter of *Trh* (thyrotropin releasing hormone), an established GC responsive gene, was examined in both OLGs and neurons. As shown in Fig 6*D*, MP induced GR binding to GRE to a similar extent in both cell types. These findings suggest that co-recruitment of HIF-1α and GR to the *Epo* promoter/enhancer occurs in OLGs but not in neurons. The association of GR and HIF-1α with the *Epo* promoter and enhancer in OLGs appears sequential occurring at 1 h and 3 h respectively following MP treatment.

The transcriptional activation of *Epo* gene by HIF-1α requires a DNA looping mechanism that brings the HIF-1α/ARNT-bound 3' enhancer to contact the promoter of *Epo* gene by interacting with CBP/p300 (Ebert and Bunn, 1998), which serves as a co-adaptor to activate a RNA polymerase II-containing general transcription factor complex (GTFs) to initiate gene transcription (von Mikecz et al., 2000). We conducted anti-CBP antibody-based ChIP assay to examine whether MP treatment recruits CBP/p300 to the *Epo* promoter in OLGs and neurons. Fig. 6*E* shows that CBP association with the *Epo* promoter was increased by approximately 6-fold after MP treatment for 1 h in OLGs but not neurons, when HIF-1α and GR were detected in the same promoter region (Fig. 6*B*). This finding

suggests that MP-induced HIF-1α-GR association with the *Epo* gene promoter is accompanied by the recruitment of CBP/p300 to the promoter to trigger *Epo* gene transcription.

Structural analysis of interactions among GR, HIF-1α, ARNT, and DNA.

To understand the interaction of the HIF-1α PAS domain with the GR-DBD and their relationship with the interaction of HIF-1α and ARNT, which is required for HIF-1α binding to HRE, we applied a computational modeling approach to perform a structural analysis of protein-protein interactions among GR, HIF-1α, and ARNT. The structures of the GR-DBD and GR-LBD were retrieved from protein data banks, 1GDC and 1NHZ, respectively, while those of HIF-1α were predicted by homology to obtain a structure for the PAS-B domain at a.a. 238-346. Based on thermal dynamics and intermolecular distances, the modeling results show that the GR-DBD binds HIF-1α on the PAS-B domain while retaining its DNA binding activity (Fig. 7*A*). Furthermore, the 7 residues of the GR-DBD (Gly470, His472, Tyr497, Arg498, Gln502, Asn506 and Glu508) interact with Tyr277, Asp250, Leu249, Glu334, Asn330, Thr297 and Asp294 of the HIF-1α PAS-B domain respectively. We modeled the structure of the HIF-1α -ARNT complex (Fig. 7*B*) to explore its possible interactions with the GR-DBD. Fig. 7*C* shows that the HIF-1α PAS-B complex interacts with the GR-DBD and ARNT simultaneously, preserving the DNA binding

activity of the GR-DBD. A co-IP study shows that MP treatment increased the association of GR with both HIF-1α and ARNT (Fig. 7*C* inset). Results from the protein docking analysis are summarized in Fig 7*D*.

Discussion

Results from the present study are the first to demonstrate that MP protects OLGs against excitotoxicity via the HIF-1α/Epo/Bcl-x_L cascade in a cell type-specific manner under normoxic condition. Several lines of experimental evidence support a causal role of this cascade in MP protection against AMPA-induced excitotoxicity in OLGs. First, exogenous Epo is effective in protecting OLGs against AMPA-induced OLG death. Second, both MP and Epo share a similar cytoprotective mechanism by upregulating Bcl-x_L expression in AMPA-treated OLGs. Third, MP transactivates *Epo* expression in a GR and HIF-1α dependent manner. Fourth, the causal roles of GR, HIF-1α and Epo are substantiated by the findings that knockdown of GR, HIF-1α or Epo expression abolishes the cytoprotective action of MP in OLGs. Finally, MP activation of HIF-1α in a GR-dependent manner involving complex GR-HIF-1α interaction is supported by pHRE$_{EPO}$-Luc studies, co-IP, ChIP assays, the yeast two-hybrid analysis, and computer modeling of protein-protein interactions.

HIF-1α, as its name implies, is induced by hypoxia. Hypoxia does upregulate Epo

expression in both OLGs and neurons. However, MP upregulation of Epo and activation of HIF-1α are selective for OLGs but not neurons under normoxic condition. This is the first demonstration of cell-type specific HIF-1α activation under normoxia. Oxygen-independent HIF-1α activation may occur with the receptor for activated C kinase 1 (RACK1) playing an important role (Liu et al., 2007). It remains to be determined whether factors that regulate co-activator binding to HIF-1α, such as FIH-1 (factor inhibiting HIF), that hydroxylates HIF-1α under normoxia to block binding of CBP/p300 (Mahon et al., 2001; Hewitson et al., 2002), involves a GR mechanism. In contrast to the GR-HIF-1α cross talk under hypoxic condition in COS7 cells engaging GR-LBD (Kodama et al., 2003), the present study reveals an essential role of GR-DBD in the normoxic mode of GR-HIF-1α interaction. In addition, the binding activity of the GR-DBD to GRE in the GR- HIF-1α-ARNT complex is preserved according to the molecular modeling results (Fig. 7A and C), in agreement with the finding that mutation of single a.a. (A458T) essential for the DNA binding activity of GR failed to interrupt GR activation of HIF-1α under hypoxia condition (38). However, we noted another 7 a.a. residues of the GR-DBD interacting with 7 corresponding a.a. residues in the HIF-1α PAS-B domain. This finding raises the possibility that different binding domains (the GR-LBD under hypoxia vs. the GR-DBD under normoxia) may be separately engaged dependent on oxygen tension. Further studies are needed to differentiate GR-HIF-1α interactions under oxygen-independent vs. oxygen-

刪除: that are

註解 [u1]: I add back this part which was in our PNAS revised ms and was accepted by Reviewer 1.

dependent paradigms involving the GR-DBD and the GR-LBD respectively.

MP-induced interaction between GR and HIF-1α is noted only in OLGs, but not neurons. This cell type-specific effect of MP is reflected by preferential co-recruitment processes of HIF-1α and GR onto the *Epo* promoter/enhancer and stabilization of HIF-1α association with the *Epo* promoter/enhancer in OLGs in comparison with neurons (Fig. 6). It is noted that both *Epo* promoter and 3' enhancer regions contain no sequences with appreciable similarity with GRE (GGTACAnnnTGTTCT), suggesting that the GR-HIF-1α complex is likely to activate the *Epo* gene via binding to HRE, not GRE. A deduced model depicted in Supplementary Fig. 2 shows that MP induces interactions among GR, HIF-1α and ARNT, followed by binding to the *Epo* gene promoter and 3'enhancer along with the CBP/p300 co-adaptor to activate *Epo* gene transcription. GR interaction with HIF-1α is the key step in determining the differential MP effects in OLGs vs. neurons. This novel signaling process opens a new avenue to treat white matter diseases by targeting OLGs specifically.

GCs, primarily MP, are the mainstay in treating diseases affecting the white matter including MS and SCI. Because of the very low BBB crossing rate (Chen et al., 1996), large doses of GCs are needed for treating these diseases. High doses of GCs frequently lead to serious side effects including suppression of immune and adrenal function, loss of connective tissue and bone mass, alteration of cognitive or mental function, hypertension,

glucose intolerance and others (McDonough et al., 2008). These side effects prevent administration of MP or other GCs for extended period of time to achieve optimal therapeutic effects. Epo, a neuroprotective cytokine, has been reported to improve functional recovery in animal models of demyelinating disorders (Li et al., 2004) and SCI (Gorio et al., 2002). However, systemic administration of Epo is also associated with serious side effects including cardiovascular, hematological and other disorders (Maiese et al., 2008). A recent FDA preliminary communication suggests systemic administration of Epo in patients with acute stroke may be associated with higher 90-day mortality with death related to intracerebral hemorrhage 4-fold higher in Epo-treated patients than those in the placebo group in a clinical trial of Epo. New therapeutic strategies directed at cell type-specific upregulation of *Epo* expression are preferred to systemic Epo or MP administration.

In conclusion, this is the first demonstration that MP protection of OLGs against excitotoxic death is mediated by the HIF-1α/Epo/Bcl-x_L cascade. This MP effect is specific in OLGs, but not neurons, mainly determined by the cell-type specific interaction between GR and HIF-1α. This cell-type specific signaling process provides an important insight into advancing therapeutic strategies for white matter diseases or SCI by OLG-specific transcriptional regulation of Epo expression to avoid serious adverse side effects associated with currently available therapeutic agents.

References

Becker PB, Gloss B, Schmid W, Strahle U, Schutz G (1986) In vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone. Nature 324:686-688.

Bracken MB, Shepard MJ, Collins WF, Holford TR, Young W, Baskin DS, Eisenberg HM, Flamm E, Leo-Summers L, Maroon J, et al. (1990) A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. N Engl J Med 322:1405-1411.

Bradbury EJ, Moon LDF, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416:636-640.

Chataway J, Porter B, Riazi A, Heaney D, Watt H, Hobart J, Thompson A (2006) Home versus outpatient administration of intravenous steroids for multiple-sclerosis relapses: a randomised controlled trial. Lancet Neurol 5:565-571.

Chen TC, Mackic JB, McComb JG, Giannotta SL, Weiss MH, Zlokovic BV (1996) Cellular uptake and transport of methylprednisolone at the blood-brain barrier. Neurosurgery 38:348-354.

Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Beattie MS (1997) Apoptosis and

delayed degeneration after spinal cord injury in rats and monkeys. Nat Med 3:73-76.

Diener HC, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, Shuaib A, Ashwood T, Wasiewski W, Alderfer V, Hardemark HG, Rodichok L (2008) NXY-059 for the treatment of acute stroke: pooled analysis of the SAINT I and II trials. Stroke 39:1751-1758.

Digicaylioglu M, Bichet S, Marti HH, Wenger RH, Rivas LA, Bauer C, Gassmann M (1995) Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci U S A 92:3717-3720.

Ebert BL, Bunn HF (1998) Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein. Mol Cell Biol 18:4089-4096.

Gorio A, Gokmen N, Erbayraktar S, Yilmaz O, Madaschi L, Cichetti C, Di Giulio AM, Vardar E, Cerami A, Brines M (2002) Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. Proc Natl Acad Sci U S A 99:9450-9455.

Hashimoto M, Sun D, Rittling SR, Denhardt DT, Young W (2007) Osteopontin-deficient mice exhibit less inflammation, greater tissue damage, and impaired locomotor recovery from spinal cord injury compared with wild-type controls. J Neurosci 27:3603-3611.

Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ (2002) Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem 277:26351-26355.

Kanellopoulos GK, Xu XM, Hsu CY, Lu X, Sundt TM, Kouchoukos NT, Chan PH (2000) White matter injury in spinal cord ischemia : protection by AMPA/kainate glutamate receptor antagonism editorial comment: protection by AMPA/kainate glutamate receptor antagonism. Stroke 31:1945-1952.

Kodama T, Shimizu N, Yoshikawa N, Makino Y, Ouchida R, Okamoto K, Hisada T, Nakamura H, Morimoto C, Tanaka H (2003) Role of the glucocorticoid receptor for regulation of hypoxia-dependent gene expression. J Biol Chem 278:33384-33391.

Lee JM, Yan P, Xiao Q, Chen S, Lee KY, Hsu CY, Xu J (2008) Methylprednisolone protects oligodendrocytes but not neurons after spinal cord injury. J Neurosci 28:3141-3149.

Lee JT, Xu J, Lee JM, Ku G, Han X, Yang DI, Chen S, Hsu CY (2004) Amyloid-beta peptide induces oligodendrocyte death by activating the neutral sphingomyelinase-ceramide pathway. J Cell Biol 164:123-131.

Li W, Maeda Y, Yuan RR, Elkabes S, Cook S, Dowling P (2004) Beneficial effect of erythropoietin on experimental allergic encephalomyelitis. Ann Neurol 56:767-777.

Lin CH, Juan SH, Wang CY, Sun YY, Chou CM, Chang SF, Hu SY, Lee WS, Lee YH (2008) Neuronal activity enhances aryl hydrocarbon receptor-mediated gene expression and dioxin neurotoxicity in cortical neurons. J Neurochem 104:1415-1429.

Lipton SA (2006) Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. Nat Rev Drug Discov 5:160-170.

Liu J, Narasimhan P, Yu F, Chan PH (2005) Neuroprotection by hypoxic preconditioning involves oxidative stress-mediated expression of hypoxia-inducible factor and erythropoietin. Stroke 36:1264-1269.

Liu YV, Baek JH, Zhang H, Diez R, Cole RN, Semenza GL (2007) RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. Mol Cell 25:207-217.

Mahon PC, Hirota K, Semenza GL (2001) FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 15:2675-2686.

Maiese K, Chong ZZ, Shang YC (2008) Raves and risks for erythropoietin. Cytokine Growth Factor Rev 19:145-155.

McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP (1998) Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. Nat Med 4:291-297.

McDonough AK, Curtis JR, Saag KG (2008) The epidemiology of glucocorticoid-associated adverse events. Curr Opin Rheumatol 20:131-137.

Miller RH, Mi S (2007) Dissecting demyelination. Nat Neurosci 10:1351-1354.

Phil E et al. (2005) Final results of MRC CRASH, a randomised placebo-controlled trial of intravenous corticosteroid in adults with head injury-outcomes at 6 months. Lancet 365:1957-1959.

Schmidt J, Gold R, Schonrock L, Zettl UK, Hartung H-P, Toyka KV (2000) T-cell apoptosis in situ in experimental autoimmune encephalomyelitis following methylprednisolone pulse therapy. Brain 123:1431-1441.

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res 31:3381-3385.

Semenza GL (2009) Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology 24:97-106.

Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE (1991) Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci U S A 88:5680-5684.

Sharp FR, Bernaudin M (2004) HIF1 and oxygen sensing in the brain. Nat Rev Neurosci 5:437-448.

Siddiq A, Aminova LR, Troy CM, Suh K, Messer Z, Semenza GL, Ratan RR (2009)

Selective inhibition of hypoxia-inducible factor (HIF) prolyl-hydroxylase 1 mediates neuroprotection against normoxic oxidative death via HIF- and CREB-independent pathways. J Neurosci 29:8828-8838.

Silva M, Benito A, Sanz C, Prosper F, Ekhterae D, Nunez G, Fernandez-Luna JL (1999) Erythropoietin can induce the expression of bcl-x_L through Stat5 in erythropoietin-dependent progenitor cell lines. J Biol Chem 274:22165-22169.

Smith T, Groom A, Zhu B, Turski L (2000) Autoimmune encephalomyelitis ameliorated by AMPA antagonists. Nat Med 6:62-66.

Stys PK, Lipton SA (2007) White matter NMDA receptors: an unexpected new therapeutic target? Trends Pharmacol Sci 28:561-566.

Tsutsui S, Vergote D, Shariat N, Warren K, Ferguson SSG, Power C (2008) Glucocorticoids regulate innate immunity in a model of multiple sclerosis: reciprocal interactions between the A1 adenosine receptor and beta-arrestin-1 in monocytoid cells. FASEB J 22:786-796.

Vakser IA (1996) Low-resolution docking: prediction of complexes for underdetermined structures. Biopolymers 39:455-464.

Vitellaro-Zuccarello L, Mazzetti S, Madaschi L, Bosisio P, Gorio A, De Biasi S (2007) Erythropoietin-mediated preservation of the white matter in rat spinal cord injury. Neuroscience 144:865-877.

von Mikecz A, Zhang S, Montminy M, Tan EM, Hemmerich P (2000) CREB-binding protein (CBP)/p300 and RNA polymerase II colocalize in transcriptionally active domains in the nucleus. J Cell Biol 150:265-274.

Wang CY, Liang YJ, Lin YS, Shih HM, Jou YS, Yu WC (2004) YY1AP, a novel co-activator of YY1. J Biol Chem 279:17750-17755.

Wrathall JR, Choiniere D, Teng YD (1994) Dose-dependent reduction of tissue loss and functional impairment after spinal cord trauma with the AMPA/kainate antagonist NBQX. J Neurosci 14:6598-6607.

Xu J, Kim GM, Ahmed SH, Yan P, Xu XM, Hsu CY (2001a) Glucocorticoid receptor-mediated suppression of activator protein-1 activation and matrix metalloproteinase expression after spinal cord injury. J Neurosci 21:92-97.

Xu J, Chen S, Ahmed SH, Chen H, Ku G, Goldberg MP, Hsu CY (2001b) Amyloid-beta peptides are cytotoxic to oligodendrocytes. J Neurosci 21:RC118.

Xu J, Chen S, Chen H, Xiao Q, Hsu CY, Michael D, Bao J (2009) STAT5 mediates antiapoptotic effects of methylprednisolone on oligodendrocytes. J Neurosci 29:2022-2026.

Zivadinov R, Rudick RA, De Masi R, Nasuelli D, Ukmar M, Pozzi-Mucelli RS, Grop A, Cazzato G, Zorzon M (2001) Effects of IV methylprednisolone on brain atrophy in relapsing-remitting MS. Neurology 57:1239-1247.

Figure Legends

Figure 1. MP upregulation of *Epo* expression in OLGs and injured spinal cord, but not neurons. ***A,*** OLGs and 10 DIV cortical neurons were treated with MP (1 μM) or $CoCl_2$ (0.4 mM) for 12 h, followed by RNA extraction. *Epo* mRNA was analyzed by RT-PCR (upper panel) and real time PCR (lower panel), in which *Gapdh* and *β-actin* mRNA served as internal controls, respectively. ***B,*** Dose-response effect of MP in inducing Epo expression in OLGs was measured by ELISA at 24 hr. ***C,*** MP (30 mg/kg body weight, i.v.) was given 30 min after SCI. The spinal cord tissues harvested 4 h and 24 h after SCI were subjected to quantitative RT-PCR for assessing the extent of *Epo* mRNA expression. The Ct value was normalized and expressed relative to the sham-operated control (Sham). Data are expressed as means ± SEM (n=4 in ***A*** and ***B***; n=5 in ***C***). $^*p<0.05$, $^{**}p<0.01$ versus the control (Ctrl) (***A***), the 0 μM MP group (***B***) or the SCI group (***C***) by one-way ANOVA and Newman-Keuls multiple comparison post test.

Figure 2. Epo and MP in AMPA-induced OLG death. ***A,*** OLGs were treated with AMPA (100 μM) supplemented with cyclothiazide (25 μM) (referred to as AMPA hereafter) with rhEPO (4 U/ml, 40 ng/ml) (left panel) or MP (right panel) for 24 h followed by Western blotting for Bcl-x_L. ***B,*** rhEPO effect on OLG viability following AMPA treatment based on MTT assay. ***C,*** *Epo* mRNA expression following OLG transfection with scrambled RNA

(Scr) or siRNA specific for rat *Epo* mRNA (si*Epo*) (upper panel). Effects of Scr or si*Epo* transfection on MP protection of OLGs against AMPA cytotoxicity (lower panel). *$p<0.05$ and **$p<0.01$ versus the control group, $^{\#}p<0.05$ versus the AMPA-treated group, NS: No significant difference between the two groups (n=4).

Figure 3. MP effect on HRE_{EPO}-driven luciferase activity in OLGs. OLGs or neurons were co-transfected with $pHRE_{EPO}$-Luc and pRL-TK for 36 h prior to the respective treatments followed by the luciferase activity assay. ***A,*** OLGs and neurons were with or without MP (1 μM) treatment for 24 h. ***B,*** OLGs were treated with $CoCl_2$ (0.4 mM) or MP (1 μM) for 24 h. OLGs transfected with pGL2 promoter-Luc served as the vector control (vector). Note transfected OLGs are responsive to MP as well as chemical hypoxia induced by $CoCl_2$. ***C,*** OLGs were treated with steroids including MP, dexamethasone (Dex), aldosterone (Ald), and 17-β-estradiol (E2), 1 μM each for 24 h. ***D,*** OLGs were pre-treated with 1 μM RU486 (RU, a GR antagonist) or spironolactone (SP, a MR antagonist) for 30 min, followed by MP (1 μM) for 24 h. ***E,*** OLGs were pre-treated with RU (1 μM) for 30 min followed by AMPA with or without MP. *$p<0.05$ and **$p<0.01$ versus vehicle-treated control (Ctrl) (n=4).

Figure 4. HIF-1α and GR knockdown on MP-induced HRE_{EPO} enhancer activity and *Epo/Bclx*$_L$ expression. OLGs were transfected with scrambled siRNA, siNr3c1 (for GR), or siHif1a (for HIF-1α) for 72 h, followed by: ***A***, transfection with $pHRE_{EPO}$-Luc for 24 h

and then MP (1 μM) treatment for 24 h, followed by luciferase activity assay; ***B,*** real-time PCR analysis of *Epo* mRNA; ***C,*** RT-PCR analysis of $Bcl\text{-}x_L$ mRNA; ***D,*** AMPA treatment with or without MP (1μM) for 24 h for cell death analysis by LDH release assay. $^{*}p<0.05$, $^{**}p<0.01$ versus vehicle-treated control (Ctrl), and $^{\#}p<0.05$ versus the AMPA-treated group (n=4)

Figure 5. MP activation of GR-HIF-1α interaction in OLGs, but not neurons. ***A,*** Immunoblotting of nuclear HIF-1α with or without MP treatment in OLGs and neurons. ***B,*** Nuclear fractions of OLGs and neurons were immunoprecipitated with an anti-GR antibody (IP-GR) and Western blotted with an anti-HIF-1α or anti-GR antibodies. IgG served as the loading control for the immunoprecipitated complex for quantitative analysis of band intensity. The right panel shows composite results. $^{**}p<0.01$ versus the control (0 min) group, n=3. ***C,*** Domain maps of GR and HIF-1α. Major domains of GR include AF-1 activation domain, DBD and LBD; major domains of HIF-1α include bHLH, PAS-A, PAS-B and oxygen-dependent degradation (ODD) domain. The shaded domains in gray were used for yeast two-hybrid analysis and computer modeling for protein-protein docking. ***D,*** Yeast two-hybrid analysis of GR-HIF-1α interaction. *Left panel*, GAL4-activation domain (GAL4AD, amino acids 768-881) hybrid and GAL4-DNA–binding domain (GAL4BD, amino acids 1-147) hybrid. *Right panel*, yeast colony numbers after transformation and β-galactosidase activity (n=5). N.D.: Not detectable.

Figure 6. MP recruitment of HIF-1α and GR onto the *Epo* promoter and 3' enhancer in OLGs, but not neurons. ***A,*** Schematic representation of the rat *Epo* gene promoter and enhancer. The *Epo* enhancer contains a HIF-1α binding site (HRE). OLGs and neurons were treated with MP (1 μM) for 0, 1 and 3 h, followed by ChIP assay for the GR- and HIF-1α-*Epo* promoter and enhancer in OLGs (***B***) and neurons (***C***). ChIP assay of untreated cells with normal IgG served as a negative control in each set of experiment. ***D,*** ChIP assay of the GRE-containing *Trh* gene promoter fragment (GRE_{Trh}) served as a GR activation control. ***E,*** OLGs and neurons were treated with MP (1 μM) for 0, 1 and 3 h, followed by ChIP assay for the CBP-*Epo* promoter. *$p<0.05$ and **$p<0.01$ versus the respective group without MP treatment, n=4.

Figure 7. Computer modeling of protein-protein interactions among HIF-1α PAS-B domain, GR-DBD, and ARNT. ***A,*** Predicted structure of HIF-1α PAS-B domain docking with the GR-DBD (PDB No. 1GDC). ***B,*** HIF-1α PAS-B domain docking with the ARNT PAS-B domain. ***C,*** GR-DBD docking with the (HIF-1α PAS-B)-(ARNT PAS-B) complex. Insets in ***A***: predicted a.a. residues for GR-DBD interaction with HIF-1α PAS-B. Inset in ***C***: co-IP of GR with HIF-1α and ARNT in the nuclear protein fraction derived from MP-treated OLGs. ***D,*** Summary of the modeled protein-protein docking results.