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Microglial Integrity is Maintained by Erythropoietin Through Integration of Akt and Its Substrates of Glycogen Synthase Kinase-3β, β-Catenin, and Nuclear Factor-κB

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Abstract

Recognized as a robust cytoprotectant for multiple tissues of the hematopoietic, vascular, cardiac, and nervous systems, erythropoietin (EPO) also is considered to be an attractive therapeutic candidate to modulate inflammatory cell function and survival during neurodegenerative disorders. To this end, microglia of the central nervous system serve a complex function not only to dispense of foreign organisms and injured cells of the brain, but also to foster tissue repair and reorganization during neuronal and vascular cell insults. We therefore examined the ability of EPO to modulate microglial cell survival and the underlying signal transduction pathways that govern microglial integrity during oxygen-glucose deprivation (OGD) - induced oxidative stress. We demonstrate in the microglial cell line EOC 2 that EPO provides direct microglial protection against early and late apoptotic programs of membrane phosphatidylserine exposure and genomic DNA degradation. Furthermore, expression and activation of Akt1 is vital to the cytoprotective capacity of EPO, since pharmacological inhibition of the PI 3-K pathway or gene silencing of Akt1 expression eliminates the ability of EPO to protect microglial cells. Through Akt1 dependent mechanisms that can be abrogated through the gene silencing of Akt1, maintenance of microglial cell integrity during OGD by EPO is closely integrated with the phosphorylation and inhibition of glycogen synthase kinase-3β activity as well as the intracellular trafficking of β-catenin and nuclear factor-κB. Further work that continues to elucidate the ability of EPO to target the intricate pathways that determine inflammatory cell function and integrity may lay the ground work for new therapeutic avenues for neurodegenerative disease.

Keywords

Akt1; apoptosis; gene silencing; GSK-3β; inflammation; microglia; NF-κB; oxygen-glucose deprivation; protein kinase B; oxidative stress; phosphatidylinositide-3-kinase; phosphatidylserine; programmed cell death; RelA; siRNA

INTRODUCTION

As monocyte derived cells that reside within the central nervous system, microglia can lead a complex life that can be responsible for an intense inflammatory response to remove injured cells and debris balanced by the ability to foster reparative processes that lead to new cell

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proliferation and tissue reorganization. For example, microglia can aggravate cellular injury through the generation of free radicals and oxidative stress (Sankarapandi, *et al.*, 1998), promote the production of pro-inflammatory cytokines that may heighten cell sensitivity to free radical exposure (Combs, *et al.*, 2001), and lead to the phagocytic removal of both neurons and vascular cells (Chong, *et al.*, 2005a,Chong, *et al.*, 2004,Kang, *et al.*, 2003b). Furthermore, microglial activation has been correlated with several neurodegenerative disorders, such as Alzheimer's disease with the co-localization of microglia and amyloid plaque development (Sheng, *et al.*, 1997). On the converse side, microglia can form a barrier for the removal of foreign microorganisms from the central nervous system and promote tissue repair during neuronal and vascular cell injury (Dringen, 2005,Maiese, *et al.*, 2005a,Neumann, *et al.*, 2006).

Given the positive impact that microglia may have upon the progression or resolution of degenerative insults to the brain, it becomes essential to consider cellular pathways that serve to support microglial survival and proliferation. To this end, robust cytoprotective agents that are known to modulate inflammatory cell function may offer attractive therapeutic targets to promote microglial survival during neurodegenerative disorders. Erythropoietin (EPO) appears to fill such a need in consideration of its role during periods of cellular inflammation. EPO can modulate cytokine expression and release (Avasarala and Konduru, 2005,Savino, *et al.*, 2006) as well as regulate the ability of microglia to engulf cells during early apoptotic injury (Chong, *et al.*, 2003b,Chong, *et al.*, 2005c). EPO also has been linked to microglial dependent brain parenchyma restructuring following cerebral injury (Bernaudin, *et al.*, 1999,Chong, *et al.*, 2002b,Maiese, *et al.*, 2004) by preserving microglia integrity (Vairano, *et al.*, 2002).

EPO can function through a number of cellular signaling pathways (Maiese, *et al.*, 2005b), none being of less prominence than those involving Akt1, one of three family members of the protein kinase B family that is highly expressed in the brain (Chong, *et al.*, 2005b) and serves as a central regulator for the proliferative and protective attributes of EPO (Ghezzi and Brines, 2004,Maiese, *et al.*, 2004). Interestingly, the activation of Akt has been linked to the promotion of microglial cell proliferation (Ito, *et al.*, 2005,Kim, *et al.*, 2004,Suh, *et al.*, 2005). In order for Akt1 to enhance cell survival, Akt1 must oversee the activity of several down-stream substrates that are necessary for the support of cell survival. In particular, inhibition of glycogen synthase kinase-3β (GSK-3β) activity through its phosphorylation is required to block apoptosis (Crowder and Freeman, 2000,Papkoff and Aikawa, 1998). With the inhibition of GSK-3β activity, "anti-apoptotic" pathways can be set in motion through β-catenin. Inhibition of GSK-3β activity promotes β-catenin integrity with its translocation to the cell nucleus to enhance cell survival (Chen, *et al.*, 2001,Li, *et al.*, 2006,Papkoff and Aikawa, 1998,You, *et al.*, 2004). In addition to these pathways, cytoprotection of EPO also has been shown to require the expression and cellular trafficking of nuclear factor-κB (NF-κB) to the cell nucleus for cellular functions that foster neural stem cell growth (Shingo, *et al.*, 2001), lead to the differentiation of glial cells (Lee, *et al.*, 2004), prevent apoptotic cell death (Bittorf, *et al.*, 2001,Chong, *et al.*, 2005c,Sae-Ung, *et al.*, 2005), and possibly assist with tissue repair by microglial cells (Sanz, *et al.*, 2002).

We demonstrate that the broad and robust cytoprotective capacity of EPO for a variety of cell systems is conserved for the maintenance of genomic DNA integrity and cellular membrane asymmetry during oxygen-glucose deprivation (OGD)-induced oxidative stress in the microglial cell line EOC 2. EPO requires the activation of Akt1 not only for microglial cellular protection, but also to modulate specific downstream substrates of Akt1, since gene silencing of Ak1 protein expression eliminates the cytoprotective capacity and control of Akt1 substrate intracellular signaling by EPO. The ability of EPO to promote microglial survival is tightly

aligned with the phosphorylation and inhibition of GSK-3β activity as well as the expression and intracellular trafficking of β-catenin and NF-κB from the cytoplasm to the nucleus.

MATERIALS AND METHODS

Microglia Cell Cultures

The microglial cell line EOC 2 was obtained from American Type Culture Collection (ATTC, Manassas, VA.). Cells were maintained in Dulbecco's modified Eagle medium (ATTC, Manassas, VA), supplemented with 10% fetal bovine serum (SIGMA, St Louis, MO), 50 μg/ml penicillin and streptomycin and 20% media from the LADMAC cell line (ATCC, Manassas, VA) which contains colony stimulating factor-1 (CSF-1) secreted by LADMAC cells. Cells were seeded onto 24-well plates or 35 mm culture dishes at a density of 1.5×10^6 cells per well or 4×10^6 cells per dish.

Experimental Treatments

Oxygen-glucose deprivation (OGD)—OGD in microglial cultures was performed by replacing media with glucose-free HBSS containing 116 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L $MgSO_4$, 1 mmol/L NaH2PO_4, 0.9 mmol/L $CaCl_2$, and 10 mg/L phenol red (pH 7.4) and cultures were maintained in an anoxic environment (95% N2 and 5% CO2) at 37 °C for 6 hours or per the experimental paradigm. During both pre-paradigm applications, EPO (R&D Systems, Minneapolis, MN) application was continuous. To inhibit Akt activation (phosphorylation), the PI 3-K inhibitor wortmannin (W) (Calbiochem, La Jolla, CA) or the Akt selective inhibitor D-2,3-dieoxy-*myo* inositol 1-(R)-2-methoxy-3-(octadecyloxy) propyl hydrogen phosphate (SH-6) (Alexis, San Diego, CA) were applied to microglial cultures 1 hour prior to OGD. The glycogen synthase kinase (GSK)-3β inhibitors SB216763 [3-(2,4-Dichlorophenyl)-4-(1-methyl-*1*H-indol-3-yl)-*1*H-pyrrole-2,5-dione] (SB21) or SB415286 [3-[(3-Chloro-4-hydroxyphenyl)amino]-4-(2-nitrophenyl)-*1*H-pyrrole-2,5-dione] (SB41) (Tocris, Ellisville, MO) were applied continuously to the microglial cultures 1 hour prior to OGD.

Assessment of Microglial Cell Survival

Microglial cell injury was determined by bright field microscopy using a 0.4% trypan blue dye exclusion method 24 hours following treatment with OGD per our previous protocols (Chong, *et al*., 2005c). The mean survival was determined by counting eight randomly selected non-overlapping fields with each containing approximately 10-20 cells (viable + non-viable). Each experiment was replicated 6 times independently with different cultures.

Assessment of DNA Fragmentation

Genomic DNA fragmentation was determined by the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay (Chong, *et al.*, 2002a,Kang, *et al.*, 2003b). Briefly, microglial cells were fixed in 4% paraformaldehyde/0.2% picric acid/0.05% glutaraldehyde and the 3′-hydroxy ends of cut DNA were labeled with biotinylated dUTP using the enzyme terminal deoxytransferase (Promega, Madison, WI) followed by streptavidin-peroxidase and visualized with 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA).

Assessment of Membrane Phosphatidylserine (PS) Residue Externalization

Phosphatidylserine (PS) exposure was assessed through the established use of annexin V. Per our prior protocols (Chong, *et al.*, 2004,Kang, *et al*., 2003b), a 30 μg/ml stock solution of annexin V conjugated to phycoerythrin (PE) (R&D Systems, Minneapolis, MN) was diluted to 3 μg/ml in warmed calcium containing binding buffer (10 mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$). Plates were incubated with 500

μl of diluted annexin V for 10 minutes. Images were acquired with "blinded" assessment with a Leitz DMIRB microscope (Leica, McHenry, IL) and a Fuji/Nikon Super CCD (6.1 megapixels) using transmitted light and fluorescent single excitation light at 490 nm and detected emission at 585 nm.

Gene Silencing of Akt1 with Small Interfering RNA (siRNA)

Cells were plated into 35 mm dishes or 24-well plates. To silence Akt1 gene expression, commercial reagents using the SMARTpool Akt1 siRNA kit (Upstate, Lake Placid, NY) were used. Transfection of siRNA duplexes were performed with Oligofectamine reagent according to manufacturer guidelines (Invitrogen, Carlsbad, CA). Experimental assays were performed 72 hours post-transfection. For each siRNA assay, negative controls contain multiple siRNAs including the target siRNA and positive controls are absent of the target siRNA.

Expression of Phosphorylated Akt1, Phosphorylated β-Catenin, and Phosphorylated Glycogen Synthase Kinase

Cells were homogenized and following protein determination, each sample (50 μg/lane) was then subjected to 7.5% SDS-polyacrylamide gel electrophoresis. After transfer, the membranes were incubated with a rabbit polyclonal antibody against a mouse monoclonal antibody against phosphorylated Akt1 (p-Akt1, Ser^{473}, 1:1000, Active Motif, Carlsbad, CA), a rabbit antibody against phosphorylated-β-catenin (1:1000) (p-β-catenin, $Ser^{33/37}$ Thr^{41}, Cell Signaling, Beverly, MA), or a rabbit antibody against phosphorylated GSK-3β (p-GSK-3β, Ser^{9}). Following washing, the membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (goat anti-mouse IgG, 1:2000 (p-Akt1) or goat anti-rabbit IgG, 1:15000 (p-β-catenin, p-GSK-3β). The antibody-reactive bands were revealed by chemiluminescence (Amersham Pharmacia Biotech, NJ).

β-Catenin Immunocytochemistry

For immunocytochemical staining of β-catenin, microglial cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. Cells were then incubated with rabbit anti-β-catenin 1 (1:100, Cell Signaling Technology, Beverly, MA) over night at 4°C and then with biotinylated anti-rabbit IgG (1:50, Vector laboratories) for 2 hours followed by Texas Red streptavidin (1:50, Vector laboratories) for 1 hour. Cells are washed in PBS and then stained with DAPI (Sigma, St. Louis, MO) for nuclear identification. Protein for β-catenin was imaged with fluorescence at the wavelengths of 565 nm (red) and 400 nm (DAPI nuclear staining).

Western Blot Analysis for the NF-κB p65 Family Member Expression

Cells were homogenized and following protein determination, each sample (50 μg/lane) was then subjected to 7.5% SDS-polyacrylamide gel electrophoresis. Following transfer, the membranes were incubated with primary rabbit antibody against NF-κB p65 (1:200) (Santa Cruz Biotechnologies, Santa Cruz, CA). After washing, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody (goat anti-rabbit IgG, 1:15000) (Pierce, Rockford, IL). The antibody-reactive bands were revealed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). The cytoplasmic and nuclear proteins were prepared by using NE-PER nuclear and cytoplasmic extraction reagents according to manufacture's instruction (purchased from Pierce, Rockford, IL). The expression of NF-κB p65 in nucleus and cytoplasm was determined by Western blot performed as described as above.

Statistical Analysis

For each experiment, the mean and standard error were determined. Statistical differences between groups were assessed by means of analysis of variance (ANOVA) from 6 replicate experiments with the post-hoc Student's t-test. Statistical significance was considered at $p<0.05$.

RESULTS

OGD is Toxic to Primary Microglia

In Fig. **1A**, microglial survival was significantly reduced over a 12 hour course following OGD application to 60 ± 2% (4 hours), 39 ± 3% (6 hours), 36 ± 2% (8 hours), and 31 ± 3% (12 hours) when compared with untreated control cultures (93 ± 3%, $p<0.01$). Since an OGD exposure period of 6 hours resulted in survival rate of approximately 40% (60% microglial cell loss), this duration of OGD toxicity was employed for the remainder of the experimental paradigms.

EPO Pre- and Post-Treatment Increases Microglial Survival During OGD

As shown in Fig. **1B**, no significant toxicity over a 24 hour period was present in microglia exposed to EPO alone in the concentrations of 0.001 ng/mL to 1000 ng/mL when compared with microglial survival in untreated control cultures (92 ± 3%). Administration of EPO 1 hour prior to OGD significantly increased survival with progressive increases in EPO concentration (Fig. **1C**). Yet, EPO (10 ng/mL) administration reached the maximal microglial survival (75 ± 3%, $p<0.01$), but concentrations lower than 0.1 ng/mL or higher than 10 ng/mL did not improve microglial survival during OGD (Fig. **1C**). In addition, concentrations greater than 10 ng/mL did not reduce microglial survival below the level for cultures exposed to OGD alone.

In post-treatment experiments (Fig. **1D**), microglial cells received EPO (10 ng/mL) at 2, 4, 6, and 12 hours following OGD with cell survival determined at 24 hours after OGD administration. EPO applied at 2 and 4 hours following OGD exposure significantly increased microglial survival from 35 ± 3% (OGD alone) to 61 ± 5% ($p<0.01$) and 56 ± 4% ($p<0.01$) respectively. In contrast, post-treatment with EPO at 6 and 12 hours following OGD exposure did not significantly increase microglial survival.

EPO Prevents Apoptotic Genomic DNA Fragmentation and Membrane Phosphatidylserine Exposure in Microglia During OGD

Twenty-four hours following OGD exposure, apoptosis with chromatin condensation and nuclear fragmentation in microglia cells was evident by TUNEL labeling and cellular membrane PS exposure was present annexin V labeling (Fig. **2A**). In contrast, microglia pretreated with EPO (10 ng/mL) 1 hour prior to OGD were without nuclear fragmentation or significant membrane PS exposure (Fig. **2A**). In Fig. **2B**, OGD lead to a significant increase in percent DNA fragmentation (65 ± 3%) when compared to untreated control cultures (7 ± 1%), but EPO significantly reduced DNA fragmentation to 33 ± 3% ($p<0.01$). Similarly, an increase in membrane PS exposure was observed in microglia 24 hours following OGD that reached a level of 66 ± 5% when compared to untreated control cultures of 11 ± 2% (Fig. **2B**). Microglia treated with EPO displayed a significant reduction in membrane PS externalization to 34 ± 3% at 24 hours after OGD.

Primary Microglia Rely Upon the PI 3-K Pathway and Akt1 Phosphorylation for EPO Protection Against OGD

Western blot assay was performed for phosphorylated Akt1 (p-Akt1) (activated form of Akt1) (Fig. **3**) 12 hours following OGD. For Akt1 phosphorylation, Ser^{473} was examined since

phosphorylation of Ser^{473} is considered to be a critical component for the complete activation of Akt1 (Bellacosa, *et al.*, 1998). Both OGD alone and EPO (10 ng/mL) alone independently increased the expression of p-Akt1, but EPO, either alone or in the presence of OGD, elevated p-Akt1 expression significantly to a greater degree than application of OGD alone. This increased expression of p-Akt1 by either OGD or EPO was blocked by application of wortmannin (W, 500 nM), which forms a covalent link with the lysine residue of PI 3-K (Wymann, *et al.*, 1996), and the specific Akt1 inhibitor SH-6 (20 μmol/L) (Kozikowski, *et al.*, 2003).

In Fig. **4A**, application of EPO (10 ng/mL) 1 hour prior to OGD prevented microglial membrane injury and trypan blue uptake, but this protection by EPO was lost during application of wortmannin (W, 500 nM). Quantification of these observations reveal that EPO (10 ng/mL) significantly increased microglial survival from 31 ± 3% to 80 ± 3% ($p<0.01$). Yet, co-application of wortmannin (W, 500 nM) or SH-6 (20 μmol/L) at concentrations that block activation of Akt1 through phosphorylation (Fig. **3**) during OGD with EPO (10 ng/mL) significantly reduced the ability of EPO to protect microglial cells against OGD, suggesting that microglial require the activation of Akt1 for EPO to exert its cytoprotective effects (Fig. **4B**).

In a similar manner for analysis with DNA fragmentation, administration of wortmannin (W, 500 nM) led to significant DNA fragmentation during application of EPO (10 ng/mL) 1 hour prior to OGD and negated the cytoprotective effects of EPO (Fig. **4C**). Quantification of these results further demonstrated that either wortmannin (W, 500 nM) or SH-6 (20 μmol/L) administration during OGD with EPO (10 ng/mL) significantly reduced the capacity of EPO to prevent DNA fragmentation in microglial cells during OGD, illustrating that microglial require the Akt1 activation for EPO preservation of cell survival and genomic DNA integrity (Fig. **4D**). When administered in the absence of OGD, wortmannin (W, 500 nM) or SH-6 (20 μmol/L) were not toxic to microglia, but did slightly enhance injury during OGD, suggesting that endogenous Akt1 activation provides a small level of protection during toxic insults (data not shown).

Microglial Cytoprotection by EPO is Eliminated During the Gene Silencing of Akt1

Pharmacological inhibitors, such as wortmannin or SH-6, can have their limitations in modulating the specific activity of a protein. We therefore have incorporated into our studies siRNA gene silencing of Akt1 to specifically knockdown Akt1 activity. Microglia were transfected with Akt1 siRNA and the expression of total Akt1 and p-Akt1 was observed through Western blot analysis (Fig. **5A**). Gene silencing of Akt1 during administration of EPO (10 ng/mL) with OGD exposure prevents phosphorylation of Akt1 (Figs. **5A** and **5B**). As shown in Figs. **5C** and **5D**, OGD significantly decreased microglial cell survival from 95 ± 4% to 36 ± 3% ($p<0.01$) and increased DNA fragmentation from 6 ± 2% to 66 ± 3% ($p<0.01$), but application of EPO (10 ng/mL) during OGD exposure prevented microglial injury and DNA fragmentation. Yet, protection by EPO was lost with gene silencing of Akt1 with decreased cell survival to 48 ± 3% ($p<0.01$) (Fig. **5C**) and increased DNA fragmentation to 55 ± 4% ($p<0.01$) during OGD exposure (Fig. **5D**), illustrating that activation of Akt1 is an essential component for EPO to prevent microglial cell injury and genomic DNA degradation. Transfection with siRNA for Akt1 alone did not significantly alter cell survival or DNA fragmentation when compared to controls.

EPO Phosphorylates Microglial Glycogen Synthase Kinase-3β (GSK-3β) through an Akt1 mediated pathway

We investigated the ability of EPO (10 ng/mL) to modulate phosphorylation of the Akt1 substrate GSK-3β during OGD at the conserved regulatory residue of Ser^9 (Eldar-Finkelman,

et al., 1996). Using western blot analysis, application of EPO (10 ng/mL) either alone or OGD significantly increased the phosphorylation of GSK-3β over a 12 hour course (Fig. **6**). In addition, EPO (10 ng/mL) resulted in the phosphorylation of GSK-3β to a significantly greater degree than phosphorylation of GSK-3β by OGD. However, the ability of EPO to phosphorylate GSK-3β was lost during transfection with Akt1 siRNA, suggesting the EPO blockade of GSK-3β is closely aligned to the presence and activity of Akt1 (Fig. **6**).

Inhibition of Glycogen Synthase Kinase-3β (GSK-3β) Activity Prevents Microglial cell Injury During OGD Similar to EPO

In Fig. **7A**, OGD leads to cellular injury and microglial trypan blue uptake 24 hours following OGD exposure. In contrast, application of the GSK-3β inhibitors (SB216763, (SB21), 5 μM or SB415286, (SB41), 25 μM) 1 hour prior to OGD in concentrations consistent with the current literature (Yoshimura, *et al.*, 2005) enhance cell survival and block trypan blue uptake. Further analysis in Fig. **7B** illustrates that inhibition of GSK-3β activity alone or in combination with the application of EPO (10 ng/mL) administered 1 hour prior to OGD significantly increase microglial survival when compared to OGD alone and to the same degree as during application of EPO alone or during co-administration with EPO, suggesting that EPO relies upon the inhibition of GSK-3β activity to exert microglial cytoprotection. Similarly, application of the GSK-3β inhibitors (SB216763, (SB21), 5 μM or SB415286, (SB41), 25 μM) 1 hour prior to OGD prevented microglial apoptotic DNA fragmentation (Fig. **7C**) assessed by TUNEL without evidence of a synergistic increase in protection against DNA fragmentation application of EPO alone or during co-administration of EPO (10 ng/mL) (Fig. **7D**), further supporting the premise that EPO utilizes inhibition of GSK-3β activity to prevent microglial cell injury and apoptotic demise.

EPO Employs Akt1 for the Prevention of β-Catenin Phosphorylation

Given that EPO is intimately linked to Akt1 for microglial cell protection and that β-catenin can be indirectly modulated by the Akt1 pathway through GSK-3β, we next examined the ability of EPO to modulate the phosphorylation of β-catenin following OGD exposure. Phosphorylation of β-catenin can occur sequentially with Ser^{45}, Thr^{41}, Ser^{37}, and Ser^{33}, but recent literature suggests that phosphorylation at Ser^{33}, Ser^{37}, and Thr^{41} occurs without the requirement of Ser^{45} (Wang, *et al.*, 2003). We therefore examined phosphorylation of β-catenin at $Ser^{33/37}$ and Thr^{41}. EPO (10 ng/mL either alone or during OGD prevented the expression of phosphorylated β-catenin (p-β-catenin) 12 hours following OGD. However, expression of p-β-catenin was significantly increased during OGD exposure (Figs. **8A** and **8B**). Interestingly, gene silencing of Akt1 during OGD also led to a marked increase in the expression of p-β-catenin either during application of EPO (10 ng/mL) alone or during the combined administration of EPO (10 ng/mL) and OGD (Figs. **8C** and **8D**), demonstrating that EPO employs Akt1 to block the phosphorylation of β-catenin.

EPO Maintains β-Catenin in the Nucleus of Microglia During OGD

In order for β-catenin to prevent apoptotic injury in cells, the protein must transfer to the cell nucleus and activate the transcription of its target genes that support cell survival (Chen, *et al.*, 2001,Li, *et al.*, 2006,Papkoff and Aikawa, 1998,You, *et al.*, 2004). We therefore used immunofluorescent staining for β-catenin and DAPI nuclear staining to follow the subcellular translocation of β-catenin in microglia during OGD alone and during combined administration of EPO (10 ng/mL) and OGD (Figs. **9A** and **9B**). With OGD exposure alone, significant immunofluorescent staining for β-catenin in the cytoplasm of microglia with minimal nuclear staining is present. This is evident by the ability to detect significant DAPI nuclear staining (white in color) in cells during merged OGD images since prominent β-catenin staining is not present in the nucleus (Figs. **9A** and **9B**). In contrast, during application of EPO (10 ng/mL)

with OGD, β-catenin is translocated to the nucleus with minimal cytoplasmic staining as shown by the pink nuclear regions on merged images of β-catenin and DAPI and subsequent quantification (Figs. **9A** and **9B**). In Fig. **9B**, quantification of nuclear β-catenin staining reveals a high proportion of β-catenin in the nucleus during EPO and OGD application, but with minimal β-catenin in the nucleus during OGD exposure alone.

EPO Promotes the Transfer of NF-κB p65 from the Cell Cytoplasm to the Cell Nucleus in Microglial Cells

Although each of the family members of NF-κB (p65 (RelA), RelB, and c-Rel) contains a C-terminal transactivation domain that can lead to gene transcription, significant gene activation is triggered by the p65 member that consists of two potent transactivation domains within its C terminus (Schmitz and Baeuerle, 1991). As a result, we chose to examine the effect of EPO in microglial cells upon the expression of NF-κB p65 and whether EPO could modulate the nuclear translocation of NF-κB p65 in microglia during OGD. Upon activation, NF-κB p65 translocates to the nucleus and increases the expression of many of its target genes, which function to prevent apoptosis (Chen, *et al.*, 2000,Chong, *et al.*, 2005d,Chong, *et al.*, 2005f,Li, *et al.*, 1999). We determined the expression of NF-κB p65 in the cytoplasmic and nuclear fractions separately by Western blot analysis. EPO (10 ng/mL) was applied 1 hour prior to OGD and cytoplasmic and nuclear protein was extracted at 6 hours following OGD. In cytoplasmic fractions, NF-κB p65 expression was significantly reduced during EPO (10 ng/mL) administration alone or with combined EPO (10 ng/ml) and OGD administration. In contrast, significant expression of NF-κB p65 was present in the cytoplasmic fraction during OGD administration alone (Fig. **10A**). Nuclear fractions revealed significant expression of NF-κB p65 during EPO (10 ng/ml) administration alone or with combined EPO (10 ng/ml) and OGD application, but minimal expression of NF-κB p65 during OGD alone, illustrating that EPO fosters the translocation of NF-κB p65 in microglia from the cytoplasm to the nucleus (Fig. **10B**).

DISCUSSION

As a cytoprotectant against toxic insults, EPO supports the survival of a number of cell systems that include the brain and cardiovascular system. In neuronal or retinal cells, EPO can prevent injury from glutamate excitotoxicity (Yamasaki, *et al.*, 2005), free radical exposure (Chong, *et al.*, 2003a,Chong, *et al.*, 2003c,Yamasaki, *et al.*, 2005), ischemia (Chong, *et al.*, 2003b,Chong, *et al.*, 2002a,Liu, *et al.*, 2006,Meloni, *et al.*, 2006,Wei, *et al.*, 2006,Yu, *et al.*, 2005), amyloid toxicity (Chong, *et al.*, 2005c), and dopaminergic cell injury (Demers, *et al.*, 2005,Signore, *et al.*, 2006). EPO also has an important role in the cardiac and vascular systems through the maintenance of endothelial cell function (Chong, *et al.*, 2003a,Chong, *et al.*, 2002a,van der Meer, *et al.*, 2004), modulation of cerebral vasospasm (Santhanam, *et al.*, 2005), the induction of angiogenesis (George, *et al.*, 2005,Maiese, *et al.*, 2005b,Sakamaki, 2004), the reduction in myocardial injury (Bullard, *et al.*, 2005,Parsa, *et al.*, 2003), and the promotion of cardiac remodeling (Miki, *et al.*, 2005).

Yet, of equal importance to the functional preservation of neuronal and vascular cells is the role of EPO during cellular inflammation. EPO can reduce cytokine gene expression in endothelial cells exposed to tumor necrosis factor (Avasarala and Konduru, 2005), decrease cytokine production during experimental autoimmune encephalomyelitis (Savino, *et al.*, 2006), and block primary microglial activation and proliferation during oxidative stress (Chong, *et al.*, 2003b,Chong, *et al.*, 2005c). In addition, EPO may foster the preservation of microglial cells for neuronal and vascular restructuring following cerebral injury (Bernaudin, *et al.*, 1999,Chong, *et al.*, 2002b,Maiese, *et al.*, 2004) by preventing apoptotic injury in microglia (Vairano, *et al.*, 2002). In regards to the capacity of EPO to maintain microglial

cellular integrity, we demonstrate that EPO alone is not toxic to microglia in a concentration range of 0.001 - 1000 ng/L. Application of EPO from 0.1 - 100.0 ng/L with either 1 hour pre-treatment or within 4 hours post-treatment significantly increases microglial cell survival during OGD. Maximum survival was observed at 10.0 ng/L, but concentrations of EPO less than 0.1 ng/L or greater than 100 ng/L did not enhance neuronal survival during OGD and may reflect a potential toxic side of EPO during cellular injury (Maiese, *et al.*, 2005b,Walrafen, *et al.*, 2004). It also should be noted that EPO retains its capacity to prevent early apoptotic injury with membrane PS externalization as well as later stages of apoptotic injury involving DNA fragmentation in microglia similar to other cell systems of neurovascular origin (Chong, *et al.*, 2003b,Chong, *et al.*, 2002a,Chong, *et al.*, 2005c,Parsa, *et al.*, 2003,Sharples, *et al.*, 2004).

Although EPO can rely on a variety of intracellular signals to foster cell proliferation and survival (Kilic, *et al.*, 2005,Menon, *et al.*, 2006a,Menon, *et al.*, 2006b), cytoprotection by EPO in microglial cells is determined by a series of signal transduction pathways that are initiated by Akt1. Pathways that involve Akt1 enhance cell survival during oxidative stress (Kang, *et al.*, 2003a,Kang, *et al.*, 2003b,Um and Lodish, 2006), free radical exposure (Chong, *et al.*, 2004,Wang and MacNaughton, 2005), neoplastic growth (Hardee, *et al.*, 2006), and amyloid exposure (Chong, *et al.*, 2005c,Du, *et al.*, 2004,Martin, *et al.*, 2001,Nakagami, *et al.*, 2002). In addition, the Akt pathway has been associated with the maintenance of microglial cell proliferation (Ito, *et al.*, 2005,Kim, *et al.*, 2004,Suh, *et al.*, 2005). We show that EPO can independently increase phosphorylation of Akt1 to a significantly greater degree than OGD alone. Yet, application of the PI 3-K inhibitor wortmannin or the specific Akt1 inhibitor SH-6 significantly blocked the ability of EPO to phosphorylate Akt1 and resulted in increased microglial cell injury and genomic DNA fragmentation during OGD (Fig. **4**), suggesting that EPO depends upon Akt1 activation to protect microglial cells during OGD. In addition, both wortmannin and SH-6 alone slightly worsened microglial survival and DNA fragmentation (Fig. **4**), illustrating that endogenous Akt1 activation in microglial cells during OGD may afford some protection during cellular insults. Loss of microglial cell protection with EPO during the gene silencing of Akt1 further supports our observations that Akt1 is necessary for EPO to protect against apoptosis during OGD (Fig. **5**).

Downstream of Akt1 activation is the modulation and phosphorylation of GSK-3β (Chong, *et al.*, 2005b,Chong, *et al.*, 2005e). Inhibition of GSK-3β activity through its phosphorylation can prevent apoptotic cell death (Crowder and Freeman, 2000,Papkoff and Aikawa, 1998) and has been associated with disorders of the nervous system (Chong, *et al.*, 2005d,Phiel, *et al.*, 2003,Qin, *et al.*, 2006). We show that GSK-3β becomes phosphorylated 12 hours after OGD exposure, but that the presence of EPO alone or during OGD significantly increases the phosphorylation of GSK-3β to a greater degree than OGD alone (Fig. **6**). Furthermore, inhibition of GSK-3β activity by application of either SB216763 (SB21) or SB415286 (SB41) alone or in combination with EPO during OGD significantly and to the same degree prevents microglial cell injury and apoptotic DNA fragmentation (Fig. **7**), suggesting that EPO uses inhibition of GSK-3β activity since co-application with GSK-3β inhibitors does not enhance cytoprotection by EPO in a synergistic fashion.

The integrity and ability of β-catenin to support an “anti-apoptotic” program is directly linked to whether the activation of GSK-3β and its ability to phosphorylate β-catenin that result in the ubiquitination and subsequent degradation of β-catenin is prevented (Chen, *et al.*, 2001,Li, *et al.*, 2006,Papkoff and Aikawa, 1998,You, *et al.*, 2004). Given the dependence of EPO on the inhibition of GSK-3β activity, we assessed the ability of EPO to control β-catenin phosphorylation and its intracellular trafficking during OGD. We show that phosphorylation of β-catenin is increased during OGD, but EPO alone or during OGD prevents phosphorylation of β-catenin 12 hours following OGD (Fig. **8**). The capacity of EPO to control the phosphorylation β-catenin and prevent its subsequent destruction is linked to Akt1, since gene

silencing of Akt1 expression during EPO application results in the significant phosphorylation of β-catenin during OGD. Furthermore, we illustrate that OGD alone maintains β-catenin in the cytoplasm of microglia, but EPO prevents this retention in the cytoplasm and allows the translocation of β-catenin from the cytoplasm to the nucleus (Fig. **9**).

In addition to centralized pathways such as Akt1, cytoprotection by EPO has been tied to the expression and nuclear translocation of NF-κB that occurs in erythroid progenitor cells (Sae-Ung, *et al.*, 2005) and is responsible for the removal of reactive oxygen species (Nakata, *et al.*, 2004), the blockade of apoptosis (Bittorf, *et al.*, 2001,Sae-Ung, *et al.*, 2005), and the prevention of amyloid toxicity (Chong, *et al.*, 2005c). Although not all studies support a cytoprotective role for NF-κB including those that study EPO (Matsushita, *et al.*, 2000,Xu, *et al.*, 2005), some injury models suggest that NF-κB may be necessary for reparative processes in the brain that depend upon microglial cells (Sanz, *et al.*, 2002). We have further clarified a potential role for NF-κB in microglial cells during the application of EPO by illustrating that EPO maintains the expression and integrity of NF-κB p65 in the nucleus of microglia at 6 hours following OGD. Without administration of EPO, OGD results in the loss of NF-κB p65 expression in the nucleus of microglia (Fig. **10**). In addition, EPO leads to the translocation of NF-κB p65 from the cytoplasm to the nucleus consistent with prior work that demonstrates the requirement for subcellular translocation for NF-κB p65 to activate its “anti-apoptotic program” (Chong, *et al.*, 2005c,Sae-Ung, *et al.*, 2005).

Under conditions where microglial cell proliferation and survival may form an important inflammatory cell counter-part for tissue repair during neuronal and vascular cell injury, we show that EPO can play a critical role to insure cellular integrity and prevent apoptotic injury in microglial cells. Similar to cellular systems with neurons or vascular cells, EPO increased microglial cell survival and prevented both early apoptotic programs with membrane PS exposure and late apoptotic injury involving genomic DNA degradation during oxidative stress associated with OGD. Promotion of microglial cell survival during OGD by EPO is coupled to the presence and activation of Akt1 and closely integrated with the inhibition of GSK-3β activity and the intracellular trafficking of β-catenin and NF-κB p65. Future investigations that continue to tease apart the conditions and controls for fostering microglial survival to promote neuronal and vascular function through cellular mediators as EPO may further the development of strategies against a variety of disorders in the nervous and vascular systems.

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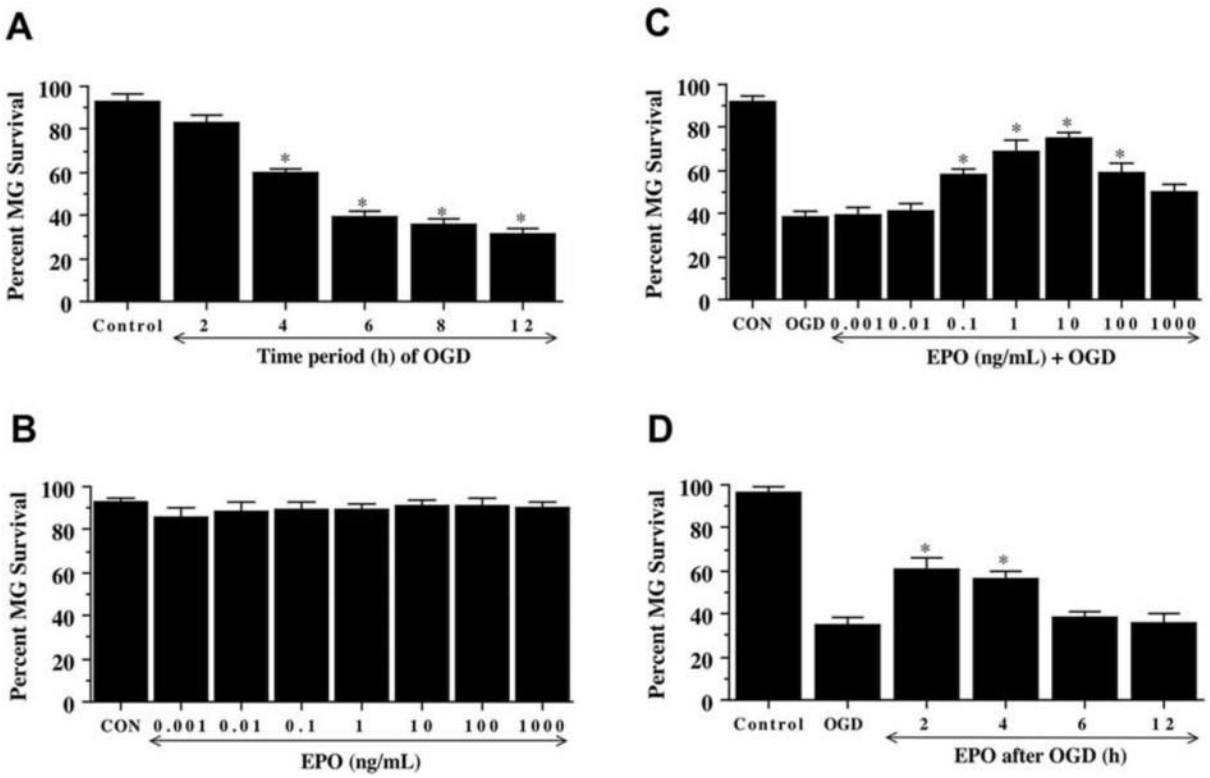

Fig (1). OGD leads to microglial cell injury that is prevented by erythropoietin (EPO)

(**A**) Microglia were exposed to OGD for 2, 4, 6, 8, and 12 hours and cell survival was determined 24 hours after OGD. The cell survival was progressively decreased over a period of 2, 4, 6, 8, and 12 hours of OGD (* $P < 0.01$ *vs.* untreated control). CON = untreated control cultures. (**B**) Increasing concentrations of EPO from 0.001 to 1000 ng/mL were applied to microglia for a 24-hour period and cell survival was determined by using the trypan blue exclusion method. The cell survival of microglia in the presence of EPO at the concentration 0.001 to 1000 ng/mL was not different from that in the cultures in absence of EPO. (**C**) EPO, at concentrations of 0.001 to 1000 ng/mL, was applied to the cultures of microglia 1 hour prior to a 6 hour period of OGD and cell survival was assessed 24 hours later. Cytoprotection by EPO during OGD was evident in cultures with 0.1 to 100 ng/mL EPO when compared with cultures exposed to OGD alone (* $P < 0.01$ *vs.* OGD). CON = untreated control cultures. (**D**) EPO (10 ng/mL) was applied to microglia at 2, 4, 6, and 12 hours following a 6 hour period of OGD exposure. Cell survival was evaluated 24 hours following OGD. Application of EPO at 2 and 4 hours after OGD significantly increased microglial survival (* $P < 0.01$ *vs.* OGD). In all cases, each data point represents the mean and SEM. Control indicates untreated cultures.

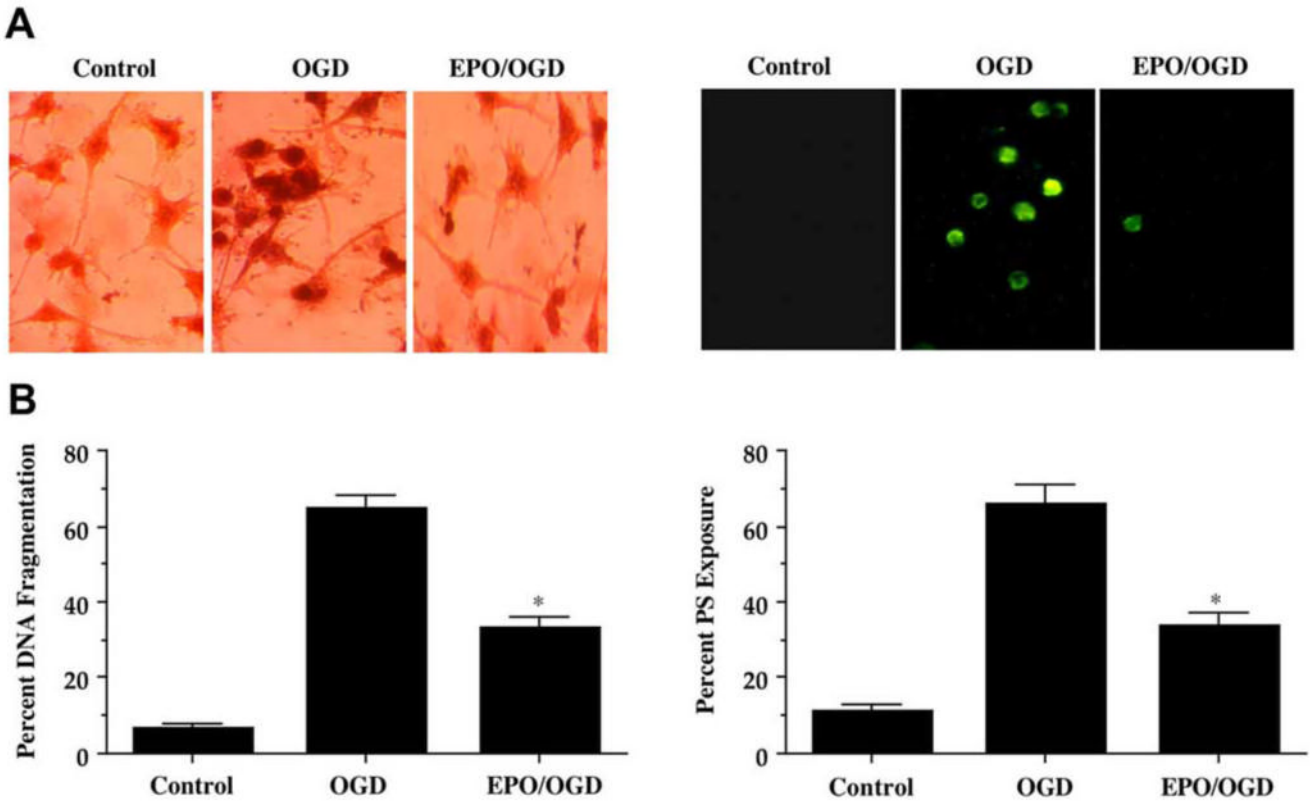

Fig (2). Erythropoietin (EPO) prevents apoptotic genomic DNA fragmentation and membrane phosphatidylserine (PS) exposure during OGD in microglia

EPO (10 ng/mL) was applied to microglia 1 hour prior to a 6 hour period of OGD exposure. Apoptotic genomic DNA fragmentation and membrane PS exposure were assessed using TUNEL assay and annexin V phycoerythrin labeling respectively 24 hours after OGD. (**A**) Representative images for TUNEL illustrate DNA fragmentation in microglia following OGD, but prevention of DNA fragmentation during EPO (10 ng/mL) application. (**B**) Quantification of data demonstrates that EPO (10 ng/mL) applied to microglia 1 hour prior to a 6 hour period of OGD significantly decreased DNA fragmentation 24 hours after OGD (* $P < 0.01$ *vs.* OGD alone). (**C**) Representative images illustrate cellular membrane PS exposure in microglia by fluorescence (F) light field with 490-nm excitation and 585-nm emission wavelengths 24 hours following OGD exposure. EPO significantly reduced PS staining during OGD exposure. (**C**) Quantification of data demonstrates that EPO (10 ng/mL) applied to microglia 1 hour prior to a 6 hour period of OGD significantly decreased membrane PS exposure 24 hours later (* $P < 0.01$ *vs.* OGD). In all cases, control indicates untreated cultures.

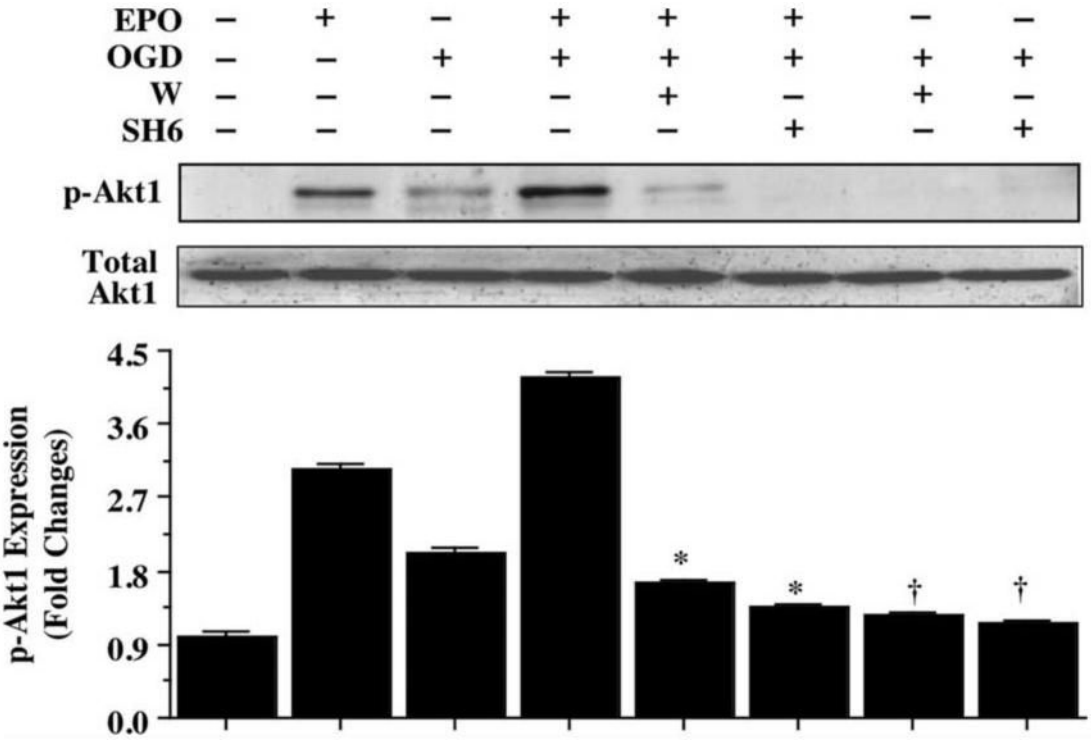

Fig (3). Erythropoietin (EPO) increases phosphorylation of Akt1 in microglia

Equal amounts of microglial protein extracts (50 μg/lane) were immunoblotted with anti-phospho-Akt1 (p-Akt1, active form) antibody 12 hours following a 6 hour period of OGD exposure. EPO (10 ng/mL) treatment significantly increased the expression of p-Akt1 alone or to a greater degree than OGD alone. In contrast, application of the phosphatidylinositol-3-kinase (PI 3-K) inhibitor wortmannin (W, 1000 nM) or the specific Akt1 inhibitor SH6 (20 μM) diminished the expression of p-Akt1 in the presence of EPO (10 ng/mL) 12 hours after OGD (* $P < 0.01$ *vs.* EPO/OGD; $^{\dagger}P < 0.01$ *vs.* OGD). Band density was performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image).

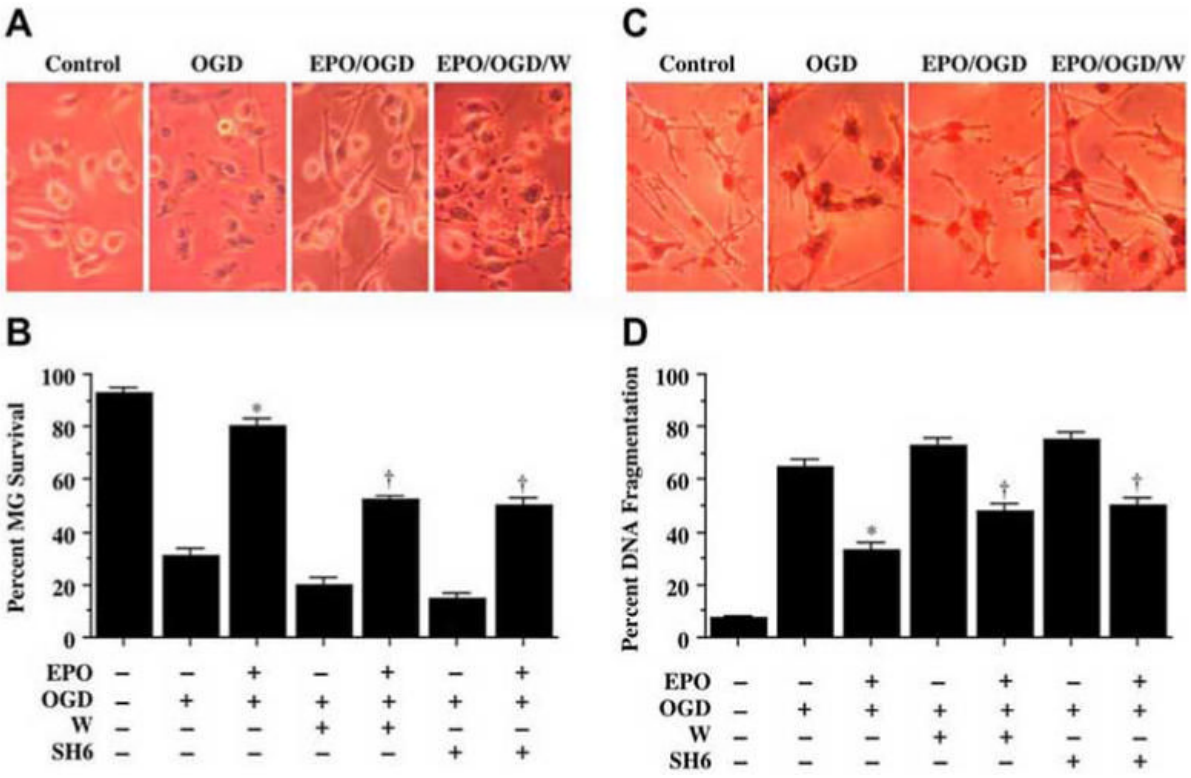

Fig (4). Erythropoietin (EPO) protects microglia against OGD through the PI 3K pathway and Akt1

EPO (10 ng/mL) was applied to microglia 1 hour prior to a 6 hour period of OGD. Cell survival and DNA fragmentation were determined 24 hours following OGD using trypan blue dye exclusion method and TUNEL assay respectively. **(A)** Representative images are illustrated for trypan blue staining in cells following OGD. EPO application significantly reduced trypan blue uptake during OGD. In contrast, co-application of the phosphatidylinositol-3-kinase (PI 3-K) inhibitor wortmannin (W, 1000 nM) with EPO prevented cytoprotection during OGD. **(B)** Quantification of data demonstrates that wortmannin (W, 1000 nM) applied 1 hour prior to OGD decreased cell survival during OGD alone and significantly blocked protection by EPO (10 ng/mL) (* $P < 0.01$ *vs.* OGD; $^{\dagger}P < 0.01$ *vs.* EPO/OGD). **(C)** Representative images illustrate DNA fragmentation in microglia following OGD. EPO (10 ng/mL) application significantly reduced TUNEL staining during OGD. In contrast, co-application of the phosphatidylinositol-3-kinase (PI 3-K) inhibitor wortmannin (W, 1000 nM) prevented EPO from blocking DNA fragmentation during OGD. **(D)** Quantification of data demonstrates that wortmannin (W, 1000 nM) applied 1 hour prior to OGD increased DNA fragmentation during OGD alone and significantly blocked the ability of EPO (10 ng/mL) to prevent DNA fragmentation (* $P < 0.01$ *vs.* OGD; $^{\dagger}P < 0.01$ *vs.* EPO/OGD). In **B** and **D**, each data point represents the mean and SEM.

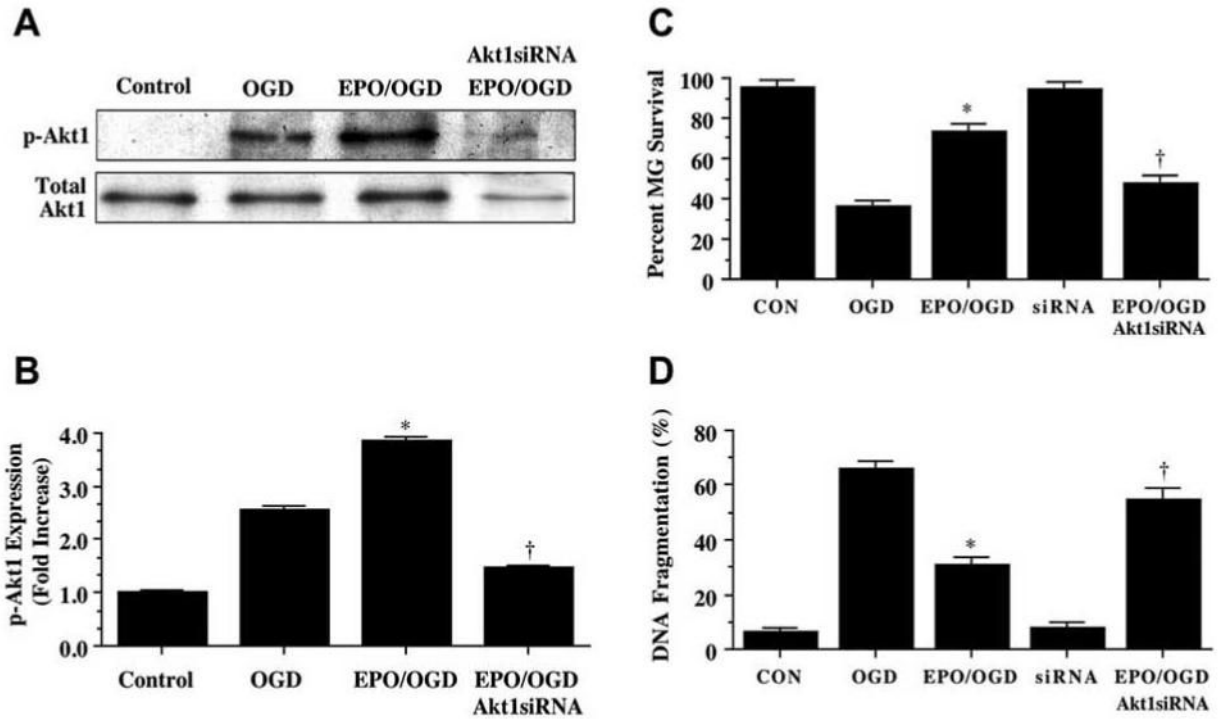

Fig (5). Gene silencing of Akt1 eliminates protection of erythropoietin (EPO) during OGD in microglia

(**A** and **B**) Equal amounts of microglial protein extracts (50 μg/lane) were immunoblotted with anti-phospho-Akt1 (p-Akt1, active form) antibody 12 hours following a 6 hour period of OGD exposure. Application of EPO (10 ng/mL) in microglia 1 hour prior to OGD significantly increased the expression of p-Akt1. Transfection of Akt1 siRNA in microglia for 72 hours prior to OGD blocked the expression of p-Akt1 (* $P < 0.01$ *vs.* OGD; $^{\dagger}P < 0.01$ *vs.* EPO/OGD). Band density was performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image). (**C** and **D**) Transfection of Akt1 siRNA in microglia for 72 hours prior to OGD was performed and cell survival and DNA fragmentation were determined 24 hours following OGD using trypan blue dye exclusion method and TUNEL assay respectively. Transfection of Akt1 siRNA in microglia prior to a 6 hour period of OGD prevented cytoprotection by EPO resulting in a significant decrease in cell survival and a significant increase in DNA fragmentation in microglia 24 hours following OGD. Akt1 siRNA alone did not alter cell survival and TUNEL staining (* $P < 0.01$ *vs.* OGD; $^{\dagger}P < 0.01$ *vs.* EPO/OGD). In all cases, each data point represents the mean and SEM. CON = untreated control cultures.

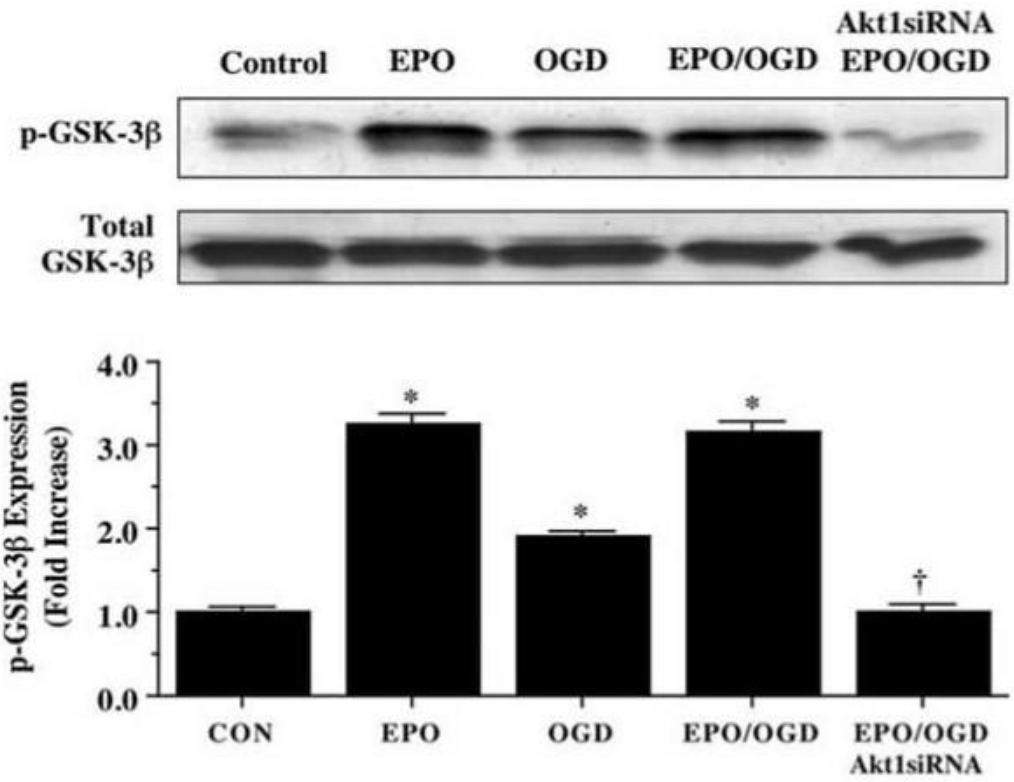

Fig (6). Erythropoietin (EPO) phosphorylates glycogen synthase kinase-3β (GSK-3β) through Akt1

Equal amounts of microglial protein extracts (50 μg/lane) were immunoblotted with phospho-GSK-3β antibody 12 hours after a 6 hour period of OGD. EPO (10 ng/mL) applied 1 hour prior to a 6 hour period of OGD significantly increased the expression of p-GSK-3β (inactive form). Transfection of Akt1 siRNA in microglia for 72 hours prior to the OGD insult decreased the efficacy of EPO resulting in a significantly diminished expression of p-GSK-3β (* $P < 0.01$ *vs.* Control; $^{\dagger}P < 0.01$ *vs.* EPO/OGD). Band density was performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image). Each data point represents the mean and SEM. CON = untreated control cultures.

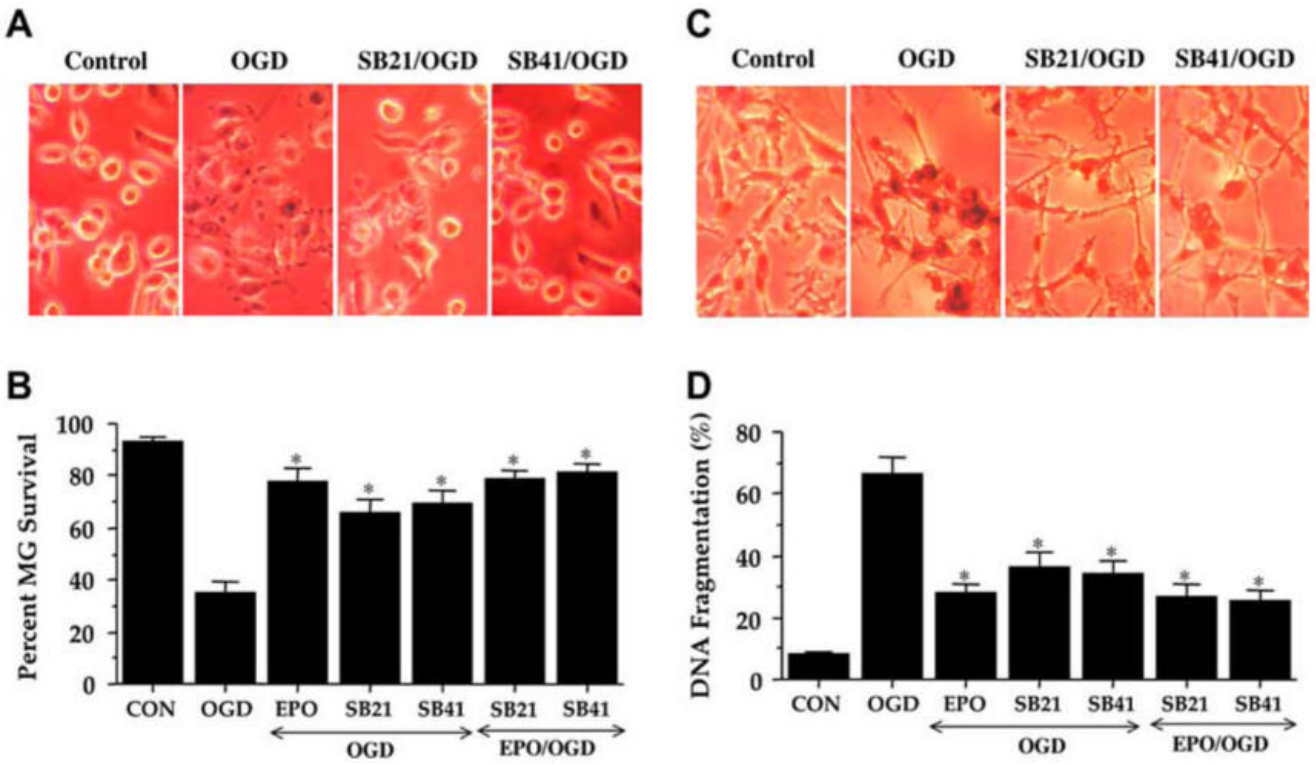

Fig (7). Inhibition of glycogen synthase kinase-3β (GSK-3β) protects microglia against OGD

The GSK-3β inhibitors SB216763 (SB21, 5 μM) or SB415286 (SB41, 25 μM) were applied to microglia 1 hour prior to a 6 hour period of OGD. Cell survival and DNA fragmentation were determined 24 hours following OGD using trypan blue dye exclusion method and TUNEL assay respectively. **(A)** Representative images illustrate trypan blue staining in microglia following OGD, but application of SB216763 (SB21, 5 μM) or SB415286 (SB41, 25 μM) significantly decreased cell staining following OGD. **(B)** Quantification of data demonstrates that SB216763 (SB21, 5 μM) or SB415286 (SB41, 25 μM) applied 1 hour prior to OGD significantly increased microglial cell survival during OGD. Furthermore, the ability of EPO (10 ng/mL) to enhance cell survival during co-application of SB216763 or SB415286 was not increased (* $P < 0.01$ *vs.* OGD). **(C)** Representative images illustrate DNA fragmentation in microglia following OGD. SB216763 (SB21, 5 μM) or SB415286 (SB41, 25 μM) application significantly prevented TUNEL staining during OGD. **(D)** Quantification of data demonstrates that SB216763 (SB21, 5 μM) or SB415286 (SB41, 25 μM) applied 1 hour prior to OGD significantly reduced DNA fragmentation during OGD, but did not further increase the ability of EPO (10 ng/mL) to block DNA fragmentation during co-application of SB216763 or SB415286 (* $P < 0.01$ *vs.* OGD). In **B** and **D**, each data point represents the mean and SEM. CON = untreated control cultures.

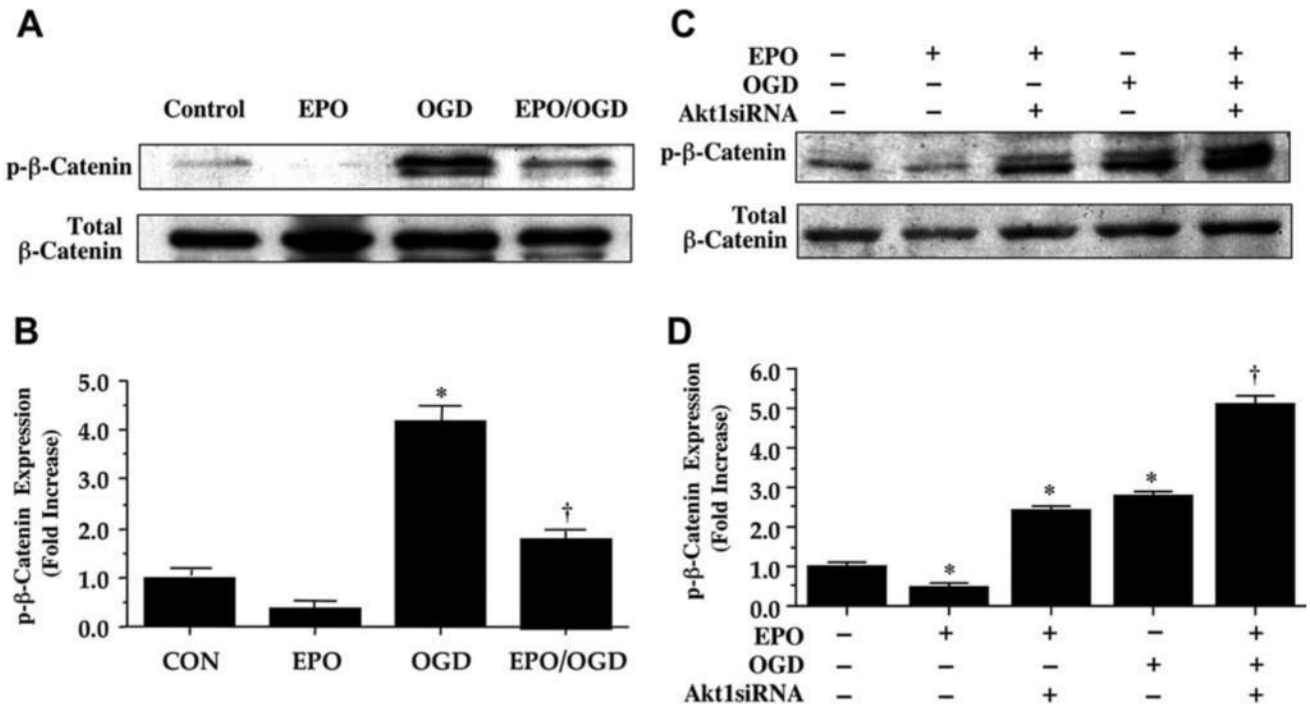

Fig (8). Erythropoietin (EPO) prevents the phosphorylation of β-catenin through an Akt1 dependent mechanism in microglia during OGD

(**A** and **B**) Equal amounts of protein extracts (50μg/lane) from microglia were immunoblotted with phospho (p)-β-catenin ($Ser^{33/37}Thr^{41}$) antibody 12 hours following a 6 hour period of OGD exposure. Although OGD exposure alone increased the expression of p-β-catenin in microglia, EPO (10 ng/mL) applied 1 hour prior to OGD was able to significantly decrease the phosphorylation β-catenin (* $P < 0.01$ *vs.* CON; $^{\dagger}P < 0.01$ *vs.* OGD). CON = untreated control cultures. (**C** and **D**) Transfection of Akt1 siRNA in microglia for 72 hours was performed prior to a 6 hour period of OGD. Subsequent protein extracts were obtained 12 hours following OGD and were immunoblotted with p-β-catenin antibody. Transfection of Akt1 siRNA was sufficient to block the ability of EPO (10 ng/mL) to prevent the expression of p-β-catenin 12 hours following OGD (* $P < 0.01$ *vs.* control untreated cultures; $^{\dagger}P < 0.01$ *vs.* OGD). Band density was performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image).

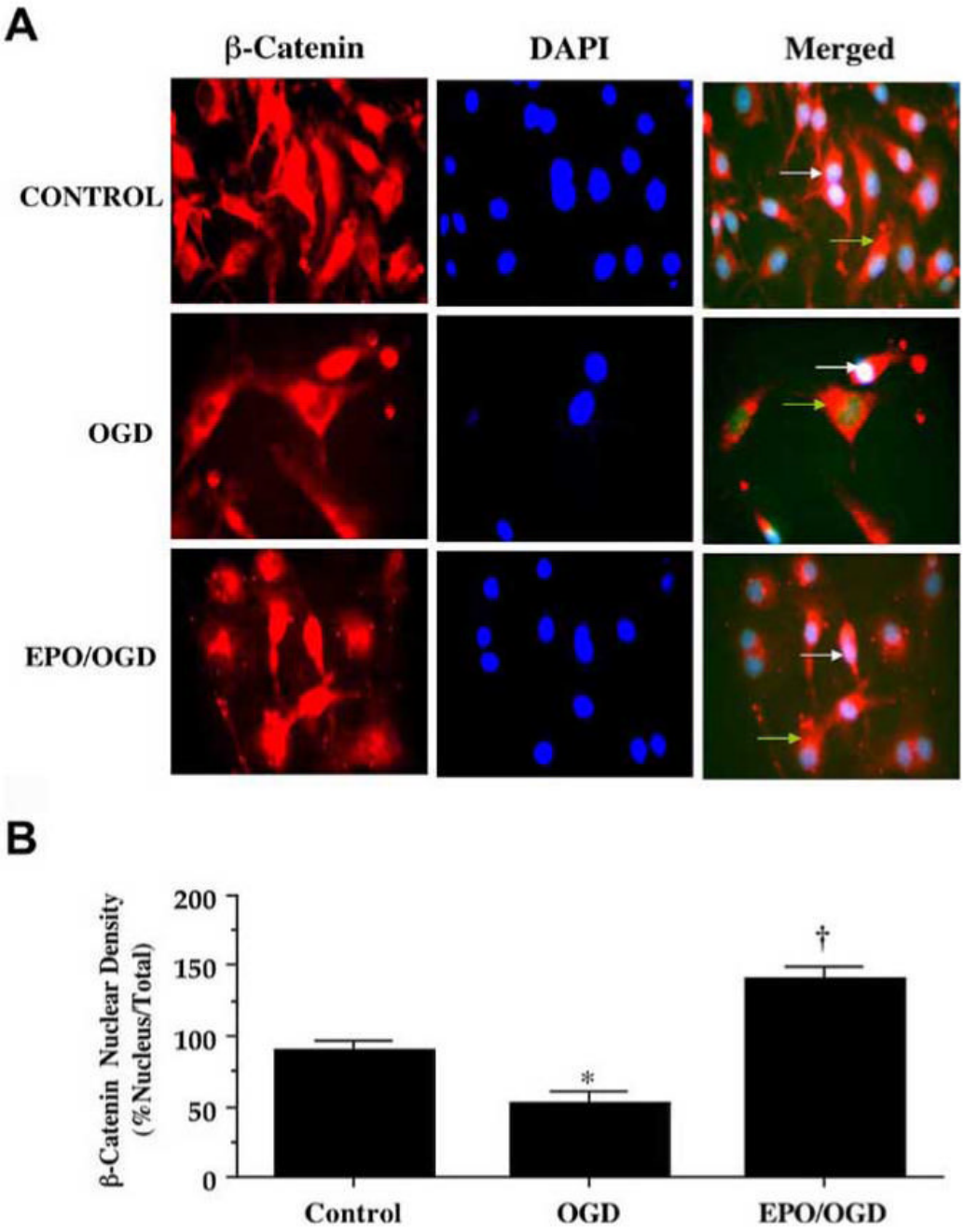

Fig (9). Erythropoietin (EPO) maintains β-catenin in the nucleus of microglia during OGD

(A) EPO (10 ng/mL) with OGD was followed at 12 hours with immunofluorescent staining with a primary rabbit anti-β-catenin antibody and visualized with Texas-red streptavidin. Microglial cell nuclei were stained with DAPI. In merged images, cells with EPO and OGD with white arrows show neuronal nuclei with strong β-catenin staining (pink) and green arrows show neuronal cytoplasm with decreased β-catenin staining in contrast to cells with OGD alone with green arrow demonstrating minimal β-catenin nuclear staining (white), illustrating the ability of EPO to promote nuclear transfer of β-catenin. **(B)** EPO increased nuclear translocation of β-catenin during OGD. Intensity of β-catenin nuclear staining was performed

using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image). Control = untreated microglia.

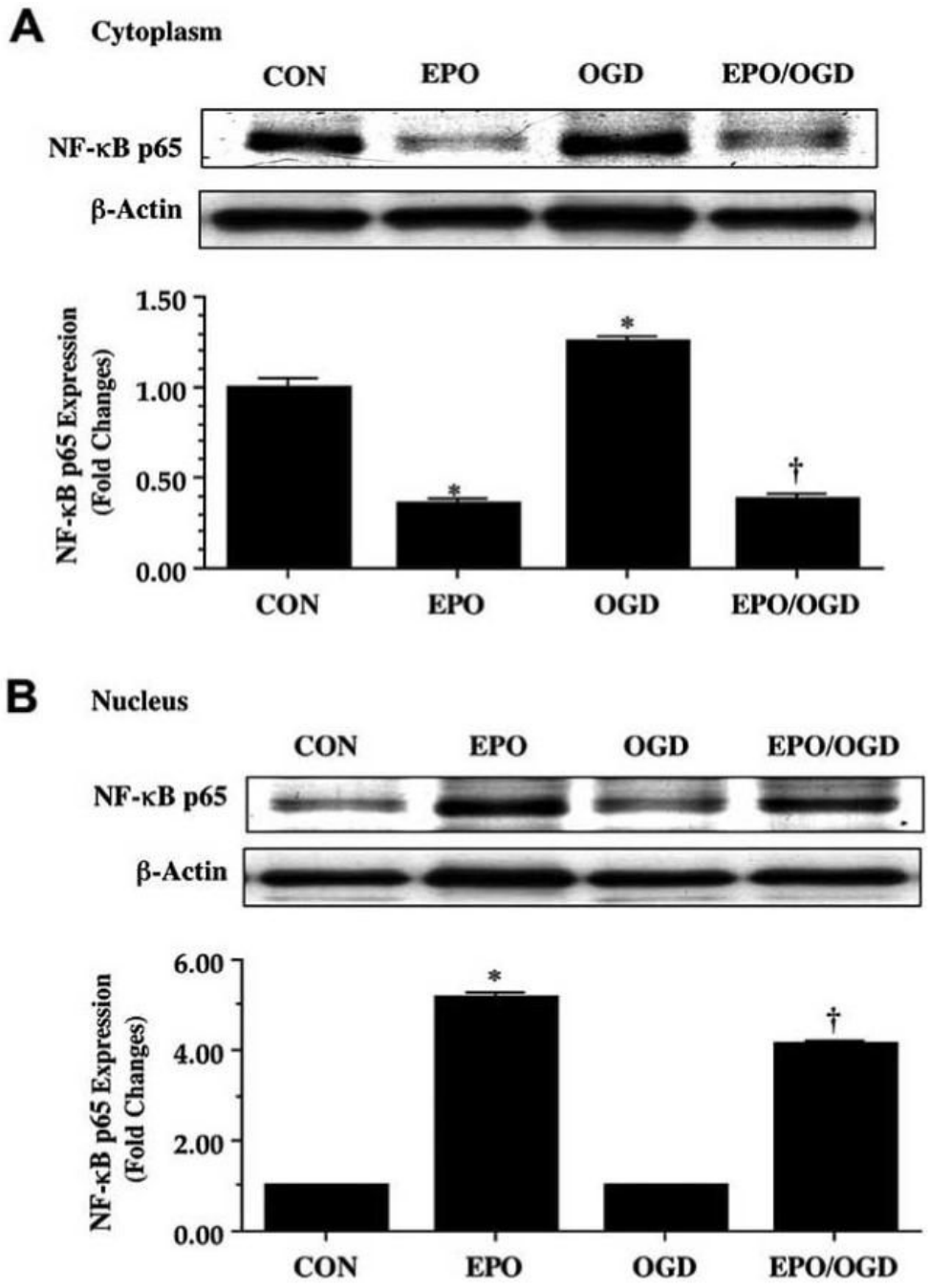

Fig (10). Erythropoietin (EPO) facilitates the nuclear translocation of NF-κB in microglia during OGD

Equal amounts of protein extracts (50 μg/lane) of the cytoplasm (**A**) and the nucleus (**B**) were immunoblotted with anti NF-κB p65 antibody 6 hours following a 6 hour period of OGD alone, an application of EPO (10 ng/mL), or administration of EPO (10 ng/mL) 1 hour prior to OGD exposure. Exposure to OGD led to the retention of NF-κB p65 in the cytoplasm of microglia (**A**), but EPO (10ng/mL) alone or during OGD facilitated the maintenance NF-κB p65 in the nucleus of microglia (**B**) (*P<0.01 *vs.* CON; †P< 0.01 *vs.* OGD). Intensity of NF-κB p65 nuclear staining was performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image). In all cases, CON = untreated control cultures.