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Erythropoietin and Wnt1 Govern Pathways of mTOR, Apaf-1, and XIAP in Inflammatory Microglia

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Abstract

Inflammatory microglia modulate a host of cellular processes in the central nervous system that include neuronal survival, metabolic fluxes, foreign body exclusion, and cellular regeneration. Elucidation of the pathways that oversee microglial survival and integrity may offer new avenues for the treatment of neurodegenerative disorders. Here we demonstrate that erythropoietin (EPO), an emerging strategy for immune system modulation, prevents microglial early and late apoptotic injury during oxidant stress through Wnt1, a cysteine-rich glycosylated protein that modulates cellular development and survival. Loss of Wnt1 through blockade of Wnt1 signaling or through the gene silencing of *Wnt1* eliminates the protective capacity of EPO. Furthermore, endogenous Wnt1 in microglia is vital to preserve microglial survival since loss of Wnt1 alone increases microglial injury during oxidative stress. Cellular protection by EPO and Wnt1 intersect at the level of protein kinase B (Akt1), the mammalian target of rapamycin (mTOR), and p70S6K, which are necessary to foster cytoprotection for microglia. Downstream from these pathways, EPO and Wnt1 control "anti-apoptotic" pathways of microglia through the modulation of mitochondrial membrane permeability, the release of cytochrome c, and the expression of apoptotic protease activating factor-1 (Apaf-1) and X-linked inhibitor of apoptosis protein (XIAP). These studies offer new insights for the development of innovative therapeutic strategies for neurodegenerative disorders that focus upon inflammatory microglia and novel signal transduction pathways.

Keywords

Akt; Apaf-1; apoptosis; cytochrome c; erythropoietin; inflammation; microglia; mitochondria; mTOR; oxidative stress; phosphatidylserine; XIAP; wingless; Wnt

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The authors have nothing to disclose.

INTRODUCTION

Inflammatory microglia oversee a broad spectrum of disorders that can affect the central nervous system. These disease processes can range from acute processes such as halting the invasion of foreign organisms [1] to limiting the progression of chronic neurodegenerative disorders such as in Alzheimer's disease [2–5]. New studies suggest that microglia may have a role during dietary oxidative stress [6], may mediate the release of neurotrophic factors during excitotoxicity [7], and also allow for regeneration and brain plasticity [8]. In a number of disorders, microglia serve an essential function to quarantine and remove non-functional neuronal and vascular cells [9–11]. Yet, potentially excessive activation of microglia also may contribute to the progression of neurodegeneration [3] and the loss of functional neuronal cells [12]. Inflammatory cells including microglia can play a critical role during the signal transduction of oxidant stress pathways [13–15] and can potentiate free radical release [16]. Given the dual modulatory role of microglia to not only confer cellular protection but also promote cellular demise in the nervous system, elucidating critical cellular mechanisms that oversee microglial survival may form a vital basis for the development of novel treatment strategies for multiple disorders of the nervous system.

In this regard, erythropoietin (EPO) has emerged as an exciting potential strategy for immune system modulation [17–24]. EPO limits leukocyte inflammation [25], prevents transplant cell loss and fosters angiogenesis [26], blocks the progression of ulcers in scleroderma [27], reduces renal inflammation during injury [28], is effective against experimental models of arthritis [29], limits immune mediated fibrosis [30], protects against pancreatic inflammation [31], and may be effective against cell injury during demyelinating disease [32]. At the cellular level, EPO is protective against tumor necrosis factor apoptosis [33], blocks cytokine gene expression [34], controls pro-inflammatory mediators [35], governs microglial activation and proliferation [10, 36, 37], and prevents the disposal of functional cells targeted by phosphatidylserine exposure [38–41]. Recently, Wnt1, a cysteine-rich glycosylated protein associated with stem cell growth, tumorigenesis and cellular senenscence [42–45] has been shown to be a vital component for the control of microglial integrity and proliferation [15, 46]. Furthermore, in neuronal cell populations, Wnt1 has been shown to prevent apoptotic neuronal injury in models of Alzheimer's disease that is medicated by EPO [47].

Here we show that during oxidant stress through oxygen-glucose deprivation (OGD) exposure, EPO governs both early apoptotic membrane PS exposure and later genomic DNA degradation in microglia through Wnt1, since blockade of Wnt1 signaling or the gene silencing of *Wnt1* eliminates the protective capacity of EPO. Loss of Wnt1 alone increases microglial injury during oxidative stress, illustrating that endogenous Wnt1 in microglia is a vital component to maintain microglial integrity. Furthermore, EPO is necessary to maintain the endogenous expression of Wnt1 in microglia that is otherwise lost during oxidant stress in the absence of EPO. Downstream from EPO and Wnt1, novel signaling through Akt1, mammalian target of rapamycin (mTOR), and p70S6K are necessary to implement protection for microglia against oxidative stress. Ultimately, EPO and Wnt1 oversee mitochondrial membrane permeability, cytochrome c release, and the expression of apoptotic protease activating factor-1 (Apaf-1) and X-linked inhibitor of apoptosis protein (XIAP) to foster microglial survival. Our work highlights the critical link between EPO and Wnt1 for the maintenance of microglia and elucidates several novel downstream therapeutic targets for inflammatory microglia that may be vital for the nervous system.

MATERIALS AND METHODS

Microglia Cell Cultures

Per our prior protocols, the microglial cell line EOC 2 was obtained from American Type Culture Collection (ATTC, Manassas, VA.) [11, 14, 15]. Cells were maintained in Dulbecco's modified Eagle medium (ATTC, Manassas, VA), supplemented with 10% heat-inactivated 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

Per our prior work, oxygen-glucose deprivation (OGD) in microglia was performed by replacing the media with glucose-free HBSS containing 116 mmol/l NaCl, 5.4 mmol/l KCl, 0.8 mmol/l $MgSO_4$, 1 mmol/l NaH_2PO_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% N_2 and 5% CO_2) at 37 °C per the experimental paradigm [11, 48, 49]. For treatments applied prior to OGD, human recombinant erythropoietin (EPO) (Sigma, St. Louis, MO), EPO blocking antibody (EPO Ab, 2 μg/ml), human recombinant Wnt1 protein (R&D Systems, Minneapolis, MN), mouse monoclonal antibody against Wnt1 (Wnt1 Ab) (1 μg/ml, R&D Systems, Minneapolis, MN), the recombinant Wnt antagonist dickkopf related protein 1 (DKK-1, 500 ng/ml, R&D Systems, Minneapolis, MN), rapamycin (RAPA, 20 nM, R&D Systems, Minneapolis, MN), or Ku 0063794 (KU, 100 nM, R&D Systems, Minneapolis, MN) were continuous.

Assessment of Cell Survival

Microglial 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 [50, 51]. 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 [52, 53]. 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

Externalization of membrane PS residues was determined by using Annexin V labeling per our prior studies [50, 51, 54, 55]. 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 [38].

Expression of Phosphorylated Akt1, Total Akt1, Phosphorylated mTOR, Total mTOR, Phosphorylated p70S6K, Total p70S6K, Apaf-1, Wnt1, GSK-3α/β, XIAP, and Cytochrome c

Cells were homogenized and each sample (50 μg/lane) was subjected to SDS-polyacrylamide gel electrophoresis [7.5% for Akt, mTOR, p70S6K, Apaf-1; 12.5% for Wnt1, GSK-3α/β, XIAP, and cytochrome c). After transfer, the membranes were incubated with a rabbit polyclonal antibody against Wnt1 (1:1000, R&D Systems, Minneapolis, MN), a rabbit monoclonal antibody against phospho-Akt1 (Ser^{473}, 1:1000 and total Akt1 (1:1000) (Cell Signaling, Beverly, MA), a rabbit antibody against total Akt1, a rabbit monoclonal antibody against phospho-mTOR (Ser^{2448}, 1:1000) and total mTOR (Cell Signaling, Beverly, MA), a rabbit antibody against phospho-p70S6K (Thr^{389}, 1:1000) and total p70S6K (1:1000) (Cell signaling Technology, Beverly, MA), a primary rabbit antibody against Apaf-1, a primary rabbit antibody against XIAP (1:1000), or a primary rabbit antibody against cytochrome c (1:1000) (Cell Signaling Technology, Beverly, MA). Following washing, the membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody goat anti-rabbit IgG (1:5000, Zymed Laboratories, Carlsbad, CA). The antibody-reactive bands were revealed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and band density was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

Akt Kinase Activity Assessment

Per our prior work [52, 56], Akt1 activity was determined by using a commercially available nonradioactive Akt1 kinase assay kit with a GSK-3β fusion protein. Cells were lysed in ice with 150 μl of lysis buffer containing 1% Triton X-100, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM Na_2PPi, and 1 mM Na_3VO4. Equal amounts of lysates (200 μg) were pre-cleared by centrifugation and pre-absorbed with protein A-protein G (1:1) agarose slurry. Immunoprecipitation was carried out over night using the immobilized anti-Akt1G1 monoclonal antibody (Cell Signaling Technology, Beverly, MA) cross-linked to agarose. Immunoprecipitates were washed three times with lysis buffer and twice with Akt kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$). Kinase assays were performed for 30 min at 30°C under continuous agitation in kinase buffer containing 200 μM ATP and 1 μg of GSK-3 fusion protein according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA). Samples were analyzed by Western blot analysis using 12.5% SDS-polyacrylamide gel and rabbit antibody against p-GSK-3α/β (Cell Signaling Technology, Beverly, MA). Data for the kinase activity are expressed as percentage of control activity.

Assessment of Mitochondrial Membrane Potential

The fluorescent probe JC-1 (Molecular Probes, Eugene, OR), a cationic membrane potential indicator, was used to assess the mitochondrial membrane potential [57, 58]. Microglia in 35 mm dishes were incubated with 2 μg/ml JC-1 in growth medium at 37 °C for 30 min. The cultures were washed three times using fresh growth medium. Mitochondria were then analyzed immediately under a Leitz DMIRB microscope (Leica, McHenry, IL, USA) with a dual emission fluorescence filter with 515–545 nm for green fluorescence and emission at 585–615 nm for red fluorescence [50].

Preparation of Mitochondria for the Analysis of Cytochrome c Release

After washing once with ice-cold PBS, cells were harvested at 10,000g for 15 min at 4°C and the resulting pellet was re-suspended in buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1

phenylmethylsulfonylfluoride) containing 250 mM sucrose and used as the mitochondrial fraction. The supernatant was subjected to ultracentrifugation at 50,000 g for 1 hour at 4 °C with the resultant supernatant used as the cytosolic fraction [59].

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 Dunnett's test. Statistical significance was considered at $P<0.05$.

RESULTS

EPO Prevents Injury in Microglia During Oxygen-Glucose Deprivation (OGD)

We examined microglial survival after exposure to OGD for the periods of 4 hours, 6 hours, 8 hours, and 12 hours. Cell survival was assessed with trypan blue exclusion method 24 hours after OGD exposure. As shown in Fig. (1**A**), representative images illustrate that OGD leads to trypan blue staining in microglia, but no significant staining is present in control cells not exposed to OGD. In Fig. (1**B**), quantitative data demonstrate that microglial survival was progressively reduced over periods following OGD application to 58 ± 4% (4 hours), 38 ± 4% (6 hours), 32 ± 4% (8 hours), and 27 ± 3% (12 hours) when compared to untreated control cultures (92 ± 3%, $p<0.01$). Since OGD exposure for a period of 6 hours resulted in survival rate of approximately 40% (a 60% microglial cell loss), this duration of OGD was used for the reminder of the experimental paradigms.

We next investigated the ability of EPO to prevent microglial cell injury following OGD exposure. EPO (0.1, 1, 10, 50 and 100 ng/ml) was given 1 hour prior to OGD and cell survival was determined 24 hours after OGD by the trypan blue dye exclusion method. As shown in Figures 1C and 1D, EPO at the concentrations of 1, 10, 50, and 100 ng/ml significantly reduced trypan blue uptake in microglia and the concentration of 10 ng/ml provided the highest cell survival for microglia, which was used for the later experiments. Concentrations lower than 0.1 ng/ml did not improve cell survival during OGD (Fig. 1**D**).

Interestingly, pre-treatment of EPO appears to provide the highest level of cell protection when applied 1 hour prior to OGD exposure with cell survival increasing from 38 ± 4% in cells exposed to OGD alone to 63 ± 5% with EPO (Fig. 1**E**). However, other pre-treatment regimens at 6, 12, and 24 hours prior to OGD also increased microglial cell survival from 38 ± 4% in microglia exposed to OGD alone to 58 ± 5%, 55 ± 4%, and 53 ± 5% respectively, but administration of EPO closest to the point of OGD exposure yielded the greatest degree of cytoprotection for microglia (Fig. 1**E**). We therefore utilized a 1 hour application of EPO prior to OGD exposure for subsequent studies.

EPO is Necessary to Offer Protection Against Apoptotic Injury in Microglia During OGD Exposure

We next investigated whether specific antagonism against exogenous EPO application with an antibody to EPO (EPO Ab) could neutralize the protective capacity of EPO. EPO Ab (2 μg/ml) in conjunction with EPO (10 ng/ml) was applied to microglial cultures 1 hour prior to a 6 hour period of OGD. Prior studies have shown that concentrations of EPO Ab of 0.50 and greater can significantly neutralize the protective capacity of EPO in other cell systems [40]. Microglial cells were exposed to OGD and either cellular genomic DNA fragmentation was assessed with TUNEL or cellular membrane PS exposure was determined by annexin V labeling method 24 hours later. In Figs. (1**F** and 1**G**), representative images demonstrate that OGD leads to DNA fragmentation and membrane PS externalization in microglia, but that

pretreatment with EPO (10 ng/ml) 1 hour prior to OGD significantly prevent DNA nuclear condensation and membrane PS exposure. Yet, inhibition of EPO with EPO blocking antibody (EPO Ab, 2 μg/ml) abrogated EPO protection for apoptotic microglial injury. Application of EPO Ab alone did not significantly alter microglial survival when compared to untreated control cultures (data not shown). Quantitative results illustrate that EPO (10 ng/ml) application significant decreased trypan blue dye uptake, DNA fragmentation, and membrane PS exposure 24 hours after OGD. Combined application of EPO with EPO Ab (2 μg/ml) significantly blocks the protective capacity of EPO resulting in a decrease in cell survival and an increase in apoptotic DNA fragmentation and PS exposure (Figs. 1**F** and 1**G**).

EPO Maintains Expression of Wnt1 During OGD

Western blot assay was performed for the endogenous cellular expression of Wnt1 at 1, 6, and 24 hours following a 6 hour period of OGD. A representative Western blot in Fig. (2**A**) demonstrates that Wnt1 expression was present within 1 hour after OGD, but the expression of Wnt1 was diminished within 6 and 24 hours following OGD exposure (Figs. 2**A** and 2**B**). Application of EPO (10 ng/ml) in microglia significantly maintained the expression of Wnt1 at 1, 6, and 24 hours after OGD exposure (Figs. 2**A** and 2**B**), suggesting that EPO can prevent the degradation of Wnt1 during OGD.

Wnt1 Prevents Microglial Injury Following OGD Exposure

Application of recombinant human Wnt1 protein (100 ng/ml) in microglia at 1, 12, 24, or 48 hours prior to OGD significantly increased cell survival as assessed by trypan blue dye uptake. The greatest degree of protection was achieved with a 1 hour pre-treatment period of Wnt1 (Fig. 2**C**). In Figs. (3**A** and 3**B**), microglial cell survival and apoptosis were assessed with trypan blue staining, apoptotic genomic DNA fragmentation (TUNEL), and membrane PS exposure (Annexin V staining) 24 hours following OGD exposure. As shown in Fig. (3**A**), representative images demonstrate that untreated control microglia have minimal trypan blue dye uptake, TUNEL staining, or Annexin V staining. In contrast, exposure to OGD leads to a significant increase in trypan blue staining, DNA fragmentation and membrane PS exposure in microglia 24 hours after OGD. Application of EPO (10 ng/ml) applied 1 hour prior to OGD significantly reduced trypan blue staining, DNA fragmentation, and membrane PS exposure. Furthermore, Wnt1 administration (100 ng/ml) alone or in combination with EPO provided a similar degree of protection as EPO alone, suggesting that EPO and Wnt1 may be dependent upon similar protective pathways (Figs. 3**A** and 3**B**).

Blockade of Wnt1 Inhibits EPO Cytoprotection in Microglia During OGD

Application of Wnt1 Ab (1 μg/ml) (Fig. 3**A**) or the Wnt antagonist DKK-1 (500 ng/ml) (Fig. 4**A**) with EPO increased cell injury, DNA fragmentation, and PS membrane exposure following OGD when compared with OGD alone, suggesting that endogenous Wnt1 activity may offer a level of protection against OGD exposure. Wnt1 Ab or DKK-1 applied to untreated control cultures were not toxic. Protection by EPO was significantly reduced during blockade of Wnt1 with Wnt1 Ab or during DKK-1 application. Quantification of results illustrate that OGD led to a significant increase in percent trypan blue staining (60 ± 4%), DNA fragmentation (59 ± 3%) and membrane PS exposure (58 ± 5%) in microglia 24 hours after OGD when compared to untreated control cultures for trypan blue staining, for DNA staining, and for PS staining respectively (Figs. 3**B** and 4**B**). Application of EPO (10 ng/ml) or Wnt1 (100 ng/ml) significantly decreased percent trypan blue staining, DNA fragmentation, and membrane PS exposure. Yet, protection with EPO was markedly reduced during Wnt1 Ab treatment or DKK-1 application (Figs. 3**B** and 4**B**).

Gene Silencing of Wnt1 Abrogates the Protective Capacity of EPO Against OGD

As shown in Figs. (5**A** and 5**B**), expression of Wnt1 was significantly decreased at 6 hours following OGD exposure. Application of EPO (10 ng/ml) prior to OGD maintained the expression of Wnt1. Yet, transfection with Wnt1 siRNA in microglia resulted in significant reduction of the expression of Wnt1 protein as revealed with Western blot analysis at 6 hours after OGD (Figs. 5**A** and 5**B**). As a control, non-specific scrambled siRNA did not alter Wnt1 protein expression in untreated control microglia or microglia exposed to OGD, demonstrating the specificity of Wnt1 siRNA to block protein expression of Wnt1.

We next assessed the ability of EPO to offer protection during gene silencing of Wnt1. As shown in Fig. (5**C**), representative images demonstrate that OGD leads to a significant increase in trypan blue staining in microglia 24 hours following OGD exposure. EPO (10 ng/ml) or Wnt1 (100 ng/ml) prevented cell injury during OGD. However, gene knockdown of *Wnt1* with siRNA significantly increased cell injury when compared with OGD alone, suggesting that endogenous Wnt1 protein is necessary for microglial protection (Fig. 5**D**). In addition, gene knockdown of *Wnt1* with siRNA during EPO application significantly reduced the protective capacity of EPO, further supporting that Wnt1 is necessary for EPO cytoprotection in microglia (Fig. 5**D**).

EPO Activates Akt1 and Requires mTOR to Protect Microglia Against OGD

Western blot assay for the cellular expression of p-Akt1 (active) was performed following OGD exposure. As shown in Fig. (6**A**), the expression of p-Akt1 was slightly increased at 6 hours following OGD exposure, but was progressively lost over a 24 hour course. Application of EPO (10 ng/ml) 1 hour prior to OGD significantly increased and maintained the expression of p-Akt1 over a 24 hour course following OGD (Fig. 6**A**). Similarly, EPO administration 1 hour prior to OGD also significantly increased and maintained the activity of Akt1 determined by the expression of p-GSK-α/β when compared to microglia exposed to OGD only (Fig. 6**B**).

Downstream from Akt1, mTOR and p70S6K are phosphorylated and activated [48, 60]. Therefore, we next investigated the ability of EPO (10 ng/ml) to alter the mTOR signaling pathway. Western blot assay for the expression of p-mTOR (active) and p-p70S6K (active) were performed following OGD. As shown in Fig. (6**C**), the expression of p-mTOR and p-p70S6K were slightly increased 6, 12, and 24 hours following OGD exposure. In contrast, treatment with EPO (10 ng/ml) prior to OGD significantly increased and maintained the expression of p-mTOR and p-p70S6K over a 24 hour period following OGD exposure (Fig. 6**C**).

Given that EPO activates the Akt1 pathways of mTOR and p70S6K, we assessed whether either EPO or Wnt1 require mTOR to foster protection of microglia during OGD. Microglial cell survival was assessed with the trypan blue dye exclusion method 24 hours following OGD exposure. Representative images demonstrate that untreated control microglia were with minimal trypan blue staining (Fig. 6**D**), but OGD leads to a significant increase in trypan blue staining in microglia 24 hours after OGD. Both EPO (10 ng/ml) and Wnt1 (100 ng/ml) prevent trypan blue uptake in microglia during OGD. However, inhibition of mTOR activity with the specific inhibitors rapamycin (RAPA, 20 nM) and Ku 0063794 (KU, 100 nM) significantly blocks protection by EPO and Wnt1 (Figs. 6**D** and 6**E**). Furthermore, combined application of EPO (10 ng/ml) and Wnt1 (100 ng/ml) failed to protect microglia in the presence of either rapamycin or Ku 0063794, supporting the premise that EPO as well as Wnt1 rely upon mTOR pathways to offer cytoprotection to microglia (Fig. 6**E**).

EPO Utilizes Wnt1 to Modulate Mitochondrial Depolarization, Cytochrome c Release, and Apaf-1 and XIAP Expression in Microglia During OGD

Using the cationic membrane potential indicator JC-1, we determined mitochondrial depolarization in microglia during OGD exposure. In Fig. (7**A**), OGD exposure produces a significant decrease in the microglia mitochondrial red/green fluorescence intensity ratio at 6 hours after OGD (53 ± 5%) when compared to untreated control mitochondria (100 ± 4%). Gene knockdown of *Wnt1* with siRNA (Fig. 7**A**) during OGD further decreased mitochondrial membrane red/green fluorescence ratio to 39 ± 5%, suggesting that endogenous Wnt1 also provides protection against mitochondrial depolarization. EPO (10 ng/ml) or Wnt1 (100 ng/ml) administration 1 hour prior to OGD significantly increased the red/green fluorescence intensity of the mitochondria to 78 ± 34% and 76 ± 4% respectively, illustrating that EPO and Wnt1 can significantly improve mitochondrial permeability transition pore membrane potential. The ability of EPO to control mitochondrial depolarization was dependent upon the presence of Wnt1 since gene knockdown of *Wnt1* with siRNA blocked the ability of EPO to prevent mitochondrial depolarization to 52 ±5% (Fig. 7**A**). Non-specific scrambled siRNA did not alter mitochondrial depolarization during OGD when compared to OGD alone (Fig. 7**A**).

In Fig. (7**B**), subsequent cytochrome c release from mitochondria was determined by Western blot for cytochrome c expression in both mitochondrial and cytosol extractions. Following OGD exposure, a significant release of cytochrome c from the mitochondria occurred indicated by increased expression of cytochrome c in cytosol. Gene knockdown of *Wnt1* further increased the release of cytochrome c into the cytosol, further supporting an endogenous protective role for Wnt1 (Fig. 7**B**). Yet, EPO (10 ng/ml) or Wnt1 (100 ng/ml) administration prevented cytochrome c release from the mitochondria. Furthermore, Wnt1 was necessary for EPO to prevent cytochrome c release from the mitochondria since transfection of Wnt1 siRNA blocked the ability of EPO to prevent cytochrome c release from the mitochondria (Fig. 7**B**).

Since mitochondrial release of cytochrome c is an important mechanism in the apoptotic cascade that can lead to caspase activation [61–66], we next examined the role of two critical components in this pathway for EPO and Wnt1, namely apoptotic protease activating factor-1 (Apaf-1) and X-linked inhibitor of apoptosis protein (XIAP). Cytochrome c in the cytoplasm binds to Apaf-1 leading to the oligomerization of Apaf-1 under the assistance of dATP/ATP and the subsequent activation of the apoptotic cascade [20]. In addition, EPO has previously been shown to prevent apoptosis through parallel pathways that prevent the induction of Apaf-1 and caspase 9 [67]. In regards to XIAP, XIAP binds to caspases such as caspase 9 to block caspase activity [2, 68]. Western blot assay was performed for Apaf-1 and XIAP (Figs. 7**C**, 8**A**, and 8**B**). At 6, 12, and 24 hours following OGD, Apaf-1 expression was significantly increased and XIAP expression was significantly decreased (Fig. 7**C**). However, EPO (10 ng/ml) (Figs. 7**C**, 8**A**, and 8**B**) or Wnt1 (100 ng/ml) (Figs. 8**A** and 8**B**) administered prior to OGD significantly reduced the expression of Apaf-1 and increased the expression of XIAP, suggesting that both EPO and Wnt1 block apoptotic downstream pathways from mitochondrial depolarization through inhibition of Apaf-1 expression and enhancement of XIAP expression. Furthermore, Wnt1 is necessary for EPO to modulate Apaf-1 and XIAP expression since application of Wnt1 Ab (1 μg/ml) with EPO blocks the ability of EPO during OGD exposure to decrease Apaf-1 expression (Figures 8A and 8B) and increase XIAP expression (Figs. 8**A** and 8**B**).

DISCUSSION

EPO has broad protective abilities in a number of cell types related to the nervous system that include neurons [10, 36, 40, 69–74], vascular cells, [39, 41, 47, 49, 67, 75–78], and non-

neuronal cells [10, 37, 74, 79]. Separately, the Wnt signaling pathway [44, 80] has been shown to protect against dopamine loss in models of Parkinson's disease [81, 82], reduce cardiomyocyte injury [83], promote hematoendothelial cell development [84], stimulate neurite extension [85], improve neurological function after injury [46], protect against amyloid neurodegeneration [56], and promote microglial integrity [15]. Interestingly, prior studies have illustrated that EPO relies upon Wnt1 to block endothelial injury in models of diabetes [38, 47].

Our present work demonstrates that Wnt1 with EPO has much broader implications in other cell types as well as models of oxidative stress with OGD. EPO maintains the expression of Wnt1 in microglial cells during oxidant stress. In addition, endogenous Wnt1 in microglia is critical to preserve microglial survival, since loss of Wnt1 alone increases microglial injury during oxidative stress. EPO requires the presence of Wnt1 in microglia to block early apoptotic PS exposure and subsequent nuclear DNA fragmentation, since blockade of Wnt1 signaling or gene silencing of *Wnt1* eliminates the protective capacity of EPO. Prevention of early apoptotic PS exposure in cells can be critical for their function and survival. If left unchecked, cells tagged by PS can be removed and destroyed by inflammatory cells during acute or chronic insults and lead to disability via loss of functional cells [11, 14, 53, 86–89]. In this regard, benefits of inflammatory cell activation during oxidative stress could be lost in the absence of the necessary signaling pathways of EPO and Wnt1.

The protective pathways of EPO and Wnt1 intersect at the level of Akt1. Akt1 controls multiple cellular mechanisms that involve processes such metabolism, vascular disease, neurodegeneration, and inflammation [1, 64, 68, 86, 90]. Under most circumstances, Akt increases cell survival, may promote tumorigenesis, but is protective against toxic insults [52, 91–96]. EPO relies upon the expression and activation of Akt1 for cytoprotection [38, 39, 97–101]. Interestingly, Wnt also has recently been shown to utilize Akt1 which previously was not considered in the Wnt signaling canonical and non-canonical pathways [38, 42, 46, 56, 83, 102–104]. Here we show that EPO and Wnt1 phosphorylate and activate Akt1 that is necessary for microglial cytoprotection during oxidative stress.

Furthermore, we demonstrate that the downstream activetion of mTOR and p70S6K by both EPO and Wnt1 are critical components for maintaining microglial integrity during oxidative stress. Prior work has shown that mTOR signaling protects neurons during oxidative stress [48, 60, 105–107], limits cardiomyocyte injury [108], may control synaptic plasticity [109], and can foster microglial activation and survival [16, 48, 60]. In addition, Wnt1 may employ mTOR to control hair follicle proliferation [110] while EPO may rely upon mTOR for bone formation [111] and to affect renal cell survival at lower concentrations [112]. Our demonstration that mTOR and p70S6K are governed by EPO and Wnt1 for microglial survival opens new possibilities for modulation of immune cell response during neurodegenerative insults.

EPO also relies upon Wnt1 to modulate mitochondrial permeability as well as the expression of Apaf-1 and XIAP in microglial cells. Loss of mitochondrial permeability leads to cytochrome c release and the induction of apoptotic and autophagic cascades [2, 61, 62, 66, 113–118]. We illustrate that both EPO and Wnt1 can block mitochondrial membrane depolarization and the release of cytochrome c, similar to prior studies with EPO [38, 41, 67, 101, 119] or Wnt1 [15, 38, 46]. However, we also demonstrate that Wnt1 is necessary for EPO to control these pathways in microglia during oxidative stress, since loss of Wnt1 during gene silencing abrogates the ability of EPO to control mitochondrial permeability and cytochrome release.

Given that EPO through Wnt1 can modulate microglial cell death though mitochondrial pathways, we also examined the role of Apaf-1 and XIAP. In vascular cells, EPO blocks cell death by preventing Apaf-1 and caspase 9 activation [67]. EPO may employ XIAP in this process since XIAP can block caspase 9 activity [2, 68] and prior work has shown an up-regulation of XIAP by EPO in renal cells [120]. We now show that EPO as well as Wnt1 in microglial cells significantly reduce Apaf-1 expression and increase XIAP expression during oxidative stress, suggesting that EPO and Wnt1 prevent the induction of microglial apoptosis not only through the control of mitochondrial membrane permeability, but also through downstream apoptotic pathways that involve Apaf-1 and XIAP. In addition, Wnt1 is required for EPO to modulate Apaf-1 and XIAP expression, since blockade of Wnt1 signaling eliminates the ability of EPO to control Apaf-1 or XIAP expression during oxidative stress.

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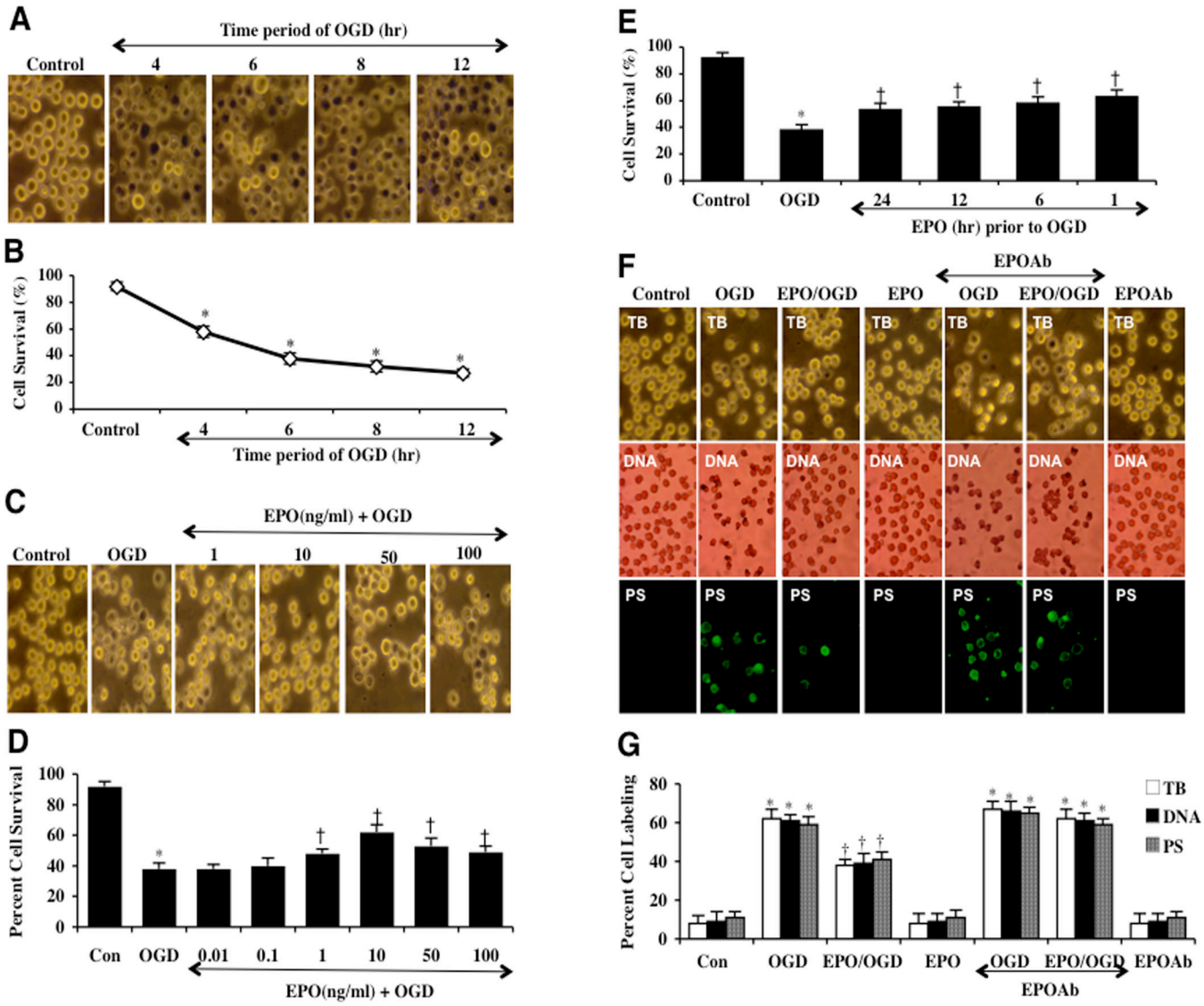

Fig. (1). EPO is necessary to foster protection in microglia during OGD exposure

(**A**) Microglia was exposed to OGD for 4, 6, 8 and 12 hours and cell survival was determined 24 hours after OGD by using trypan blue exclusion method. Representative images illustrate that OGD leads to progressively increased microglial injury over time. Control = untreated microglia. **(B)** Quantitative analysis shows that microglial survival was significantly decreased following OGD exposure when compared with untreated control cultures (**P* <0.01 *vs*. Control). Each data point represents the mean and SEM from 6 experiments. **(C** and **D)** EPO was applied to microglial cultures at the concentrations of 0.01, 0.1, 1,10, 50, and 100 ng/ml 1 hour prior to OGD and cell survival was determined 24 hours after OGD with the trypan blue dye exclusion method. Representative images show that EPO (1–100 ng/ml) significantly reduced trypan blue staining and increased cell survival following OGD (**P*<0.01 *vs*. untreated control; †*P* <0.05 *vs.* OGD). Each data point represents the mean and SEM from 6 experiments. **(E)** EPO was applied to microglial cultures 24, 12, 6 or 1 hour prior to a 6 hour period of OGD and cell survival was determined 24 hours after OGD with the trypan blue dye exclusion method. The quantitative results show that 1 hour is the most effective pretreatment time with maximal survival achieved after EPO application (**P*<0.01 *vs*. untreated control; †*P* <0.05 *vs.* OGD). Each

data point represents the mean and SEM from 6 experiments. **(F)** Representative images demonstrate that OGD led to a significant increase in trypan blue staining, DNA fragmentation, and membrane PS exposure in microglia at 24 hours after OGD compared to untreated control cultures, which was prevented by EPO (10 ng/ml) application. Yet, inhibition of EPO with an EPO blocking antibody (EPO Ab, 2 μg/ml) abrogates the efficacy of EPO on cell survival and apoptotic injury. **(G)** Quantification of the results in **F** illustrate that EPO (10 ng/ml) application significant decreased percent trypan blue uptake, DNA fragmentation, and membrane PS exposure 24 hours after OGD when compared to OGD treated alone. Inhibition of EPO with EPO Ab (2 μg/ml) diminishes the efficacy of EPO with a decrease in cell survival and an increase in percent apoptotic DNA fragmentation and PS exposure (*$P < 0.01$ *vs*. untreated control; $^{\dagger}P < 0.05$ *vs*. OGD). Each data point represents the mean and SEM from 6 experiments.

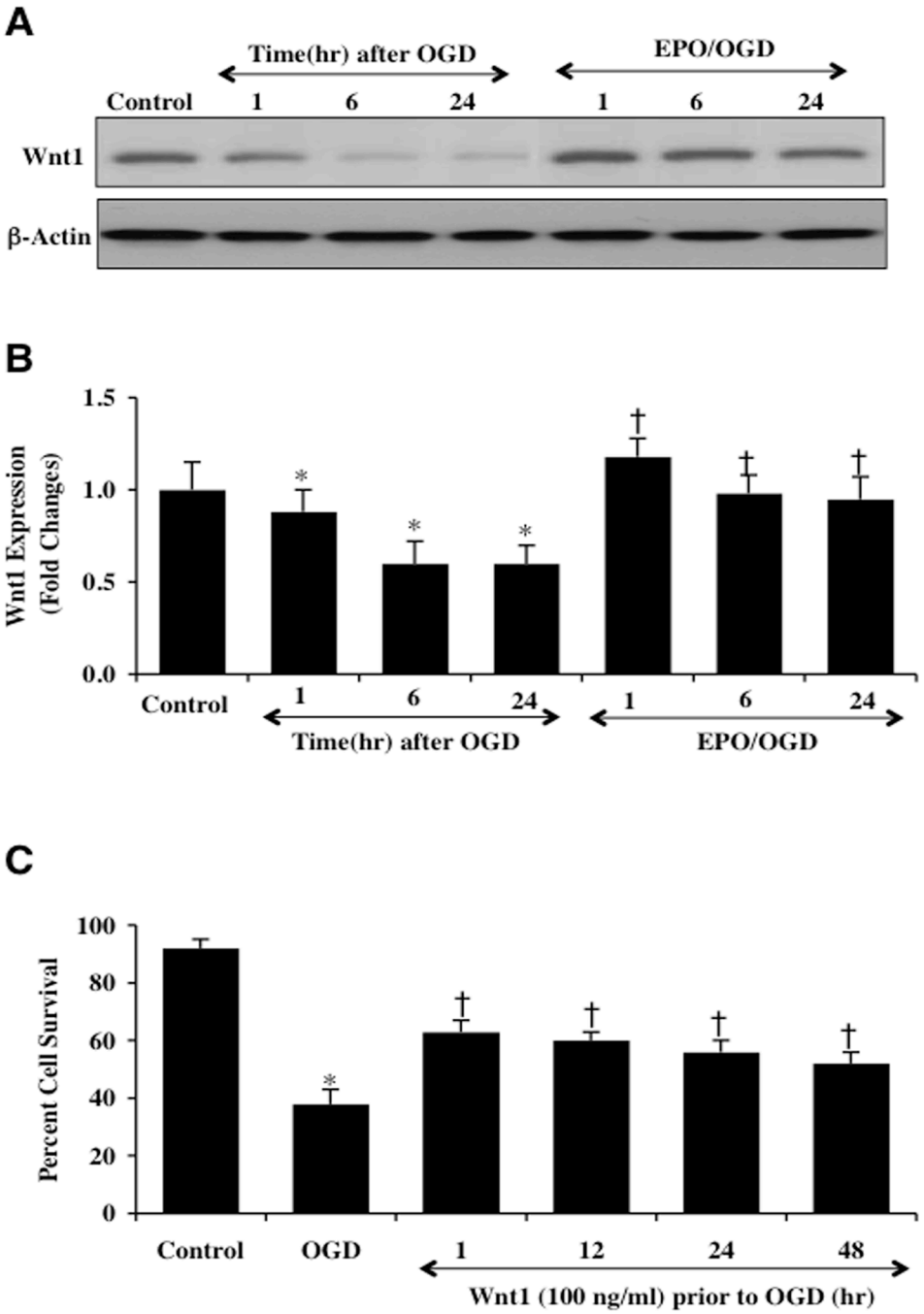

Fig. (2). EPO maintains the expression of Wnt1 and the highest microglial survival occurs with early Wnt1 application

(**A** and **B)** Microglial protein extracts (50 μg/lane) were immunoblotted with anti-Wnt1 at 1, 6 and 24 hours after OGD. Wnt1 expression was progressively reduced over 24 hours after OGD exposure (**P*<0.01 *vs*. control). In contrast, EPO (10 ng/ml) given 1 hour prior to OGD significantly increased Wnt1 expression at 1, 6 and 24 hours respectively compared with OGD alone (†*P* <0.01 *vs*. OGD). **(C)** Wnt1 was applied to microglial cultures 1, 12, 24, or 48 hours prior to a 6 hour period of OGD and cell survival was determined 24 hours after OGD with the trypan blue dye exclusion method. Wnt1 application significantly increased cell survival 24 hours following OGD with maximal efficacy with a 1 hour pretreatment

(*P<0.01 *vs*. untreated control; $^{\dagger}P$ <0.01 *vs*. OGD). Each data point represents the mean and SEM from 3 experiments.

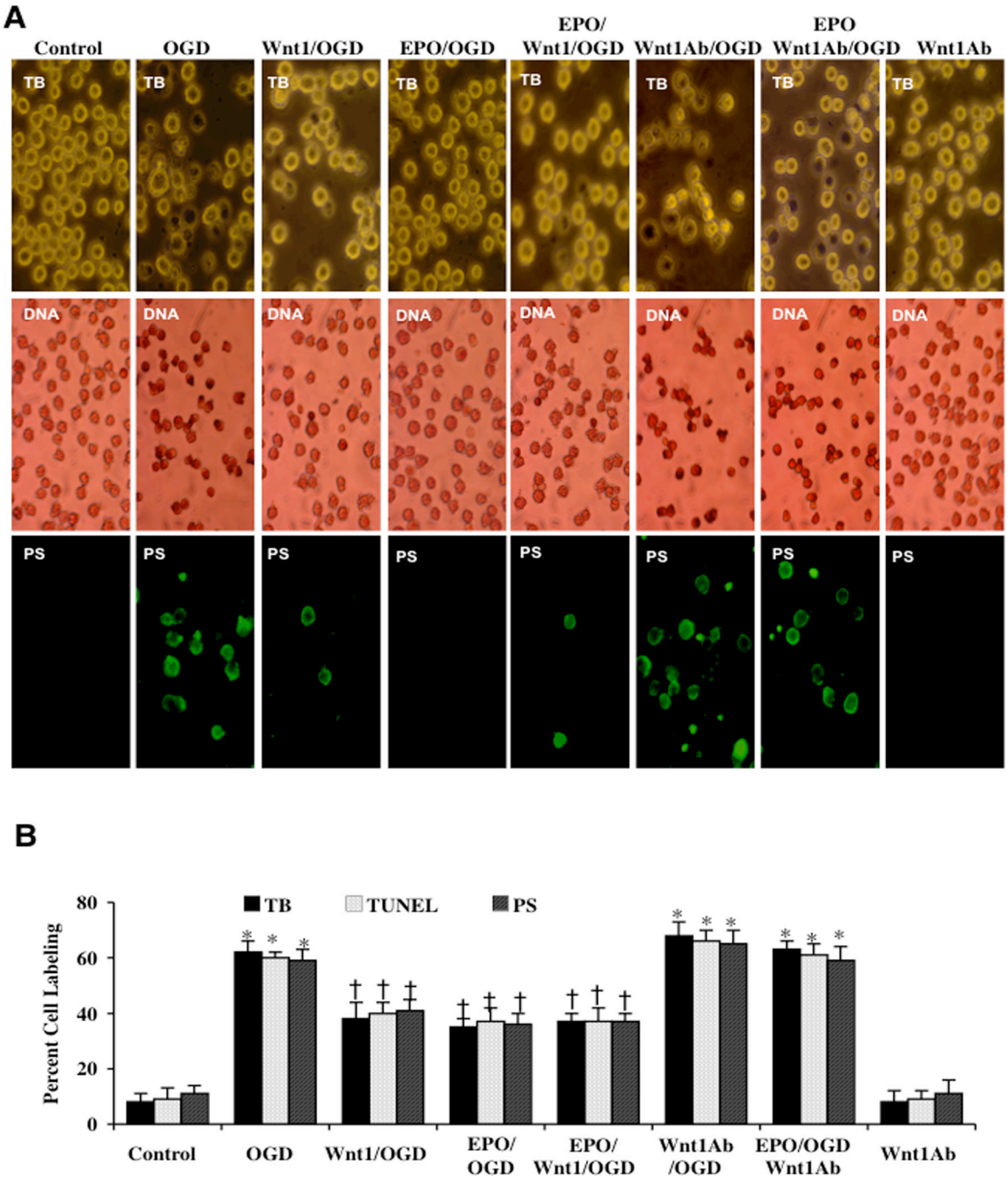

Fig. (3). Wnt1 antibody administration decreases the ability of EPO and Wnt1 to protect microglia against OGD

(A) Microglial cells were exposed to OGD for 6 hours and cell survival, DNA fragmentation, and PS exposure were determined 24 hours after OGD with the trypan blue dye exclusion method, TUNEL, and annexin V labeling method respectively. Representative images illustrate that Wnt1 (100 ng/ml) and EPO (10 ng/ml) administration during OGD significantly reduced trypan blue staining, genomic DNA degradation, and membrane PS externalization (green fluorescence). In contrast, blockade of Wnt1 with Wnt1 Ab (1 μg/ml) resulted in increased trypan blue staining, DNA fragmentation, and membrane PS exposure and also attenuated the protective ability of EPO. Combined Wnt1 and EPO application

yielded a similar protection to EPO applied only during OGD. **(B)** Quantification of data illustrates that percent trypan blue staining, DNA fragmentation, and membrane PS externalization were significantly increased following a 6 hour period of OGD when compared to untreated microglial control cultures, but Wnt1 (100 ng/ml), EPO (10 ng/ml), or EPO/Wnt1 combined therapy increased cell survival and prevented DNA fragmentation and membrane PS exposure during OGD. Inhibition of Wnt1 with Wnt1 Ab (1μg/ml) abrogates the efficacy of EPO during OGD (*$P < 0.01$ *vs*. untreated control; †$P < 0.01$ *vs*. OGD). Each data point represents the mean and SEM from 6 experiments.

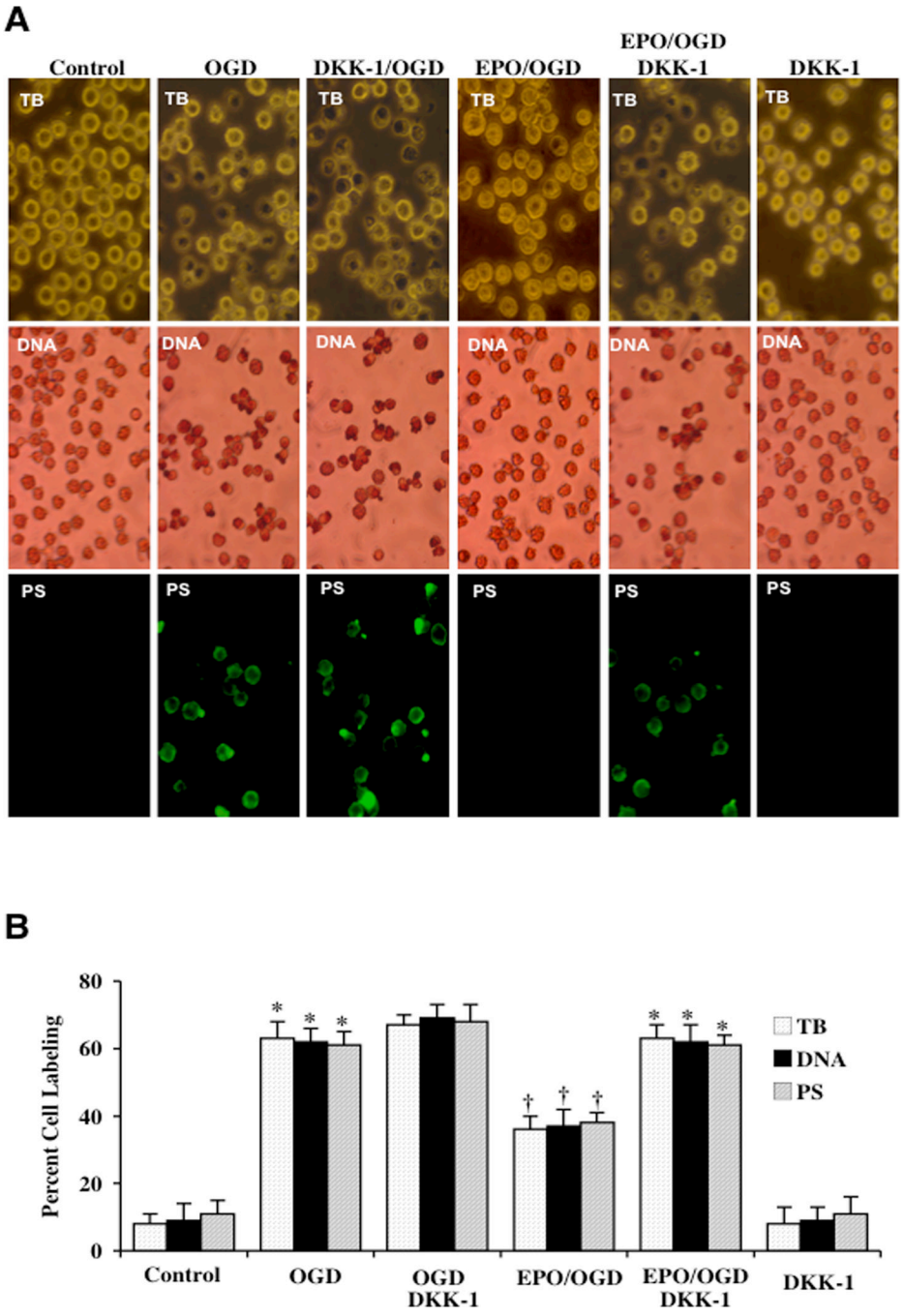

Fig. (4). Wnt antagonist DKK-1 blocks protection by EPO during OGD

(A) Microglial cells were exposed to OGD for 6 hours and cell survival, DNA fragmentation, and PS exposure were determined 24 hours after OGD with the trypan blue dye exclusion method, TUNEL, and annexin V labeling respectively. Representative images illustrate that EPO (10 ng/ml) administration during OGD significantly reduced trypan blue staining, genomic DNA degradation, and membrane PS externalization (green fluorescence). In contrast, blockade of Wnt cell signaling with DKK-1 (500 ng/ml) resulted in increased trypan blue staining, DNA fragmentation, and membrane PS exposure and also blocked protection by EPO during OGD exposure. DKK-1 (500 ng/ml) application alone to microglial cultures increased cell injury during OGD. **(B)** Quantification of data illustrates

that percent trypan blue staining, DNA fragmentation, and membrane PS externalization were significantly increased following a 6 hour period of OGD when compared to untreated microglial control cultures, but EPO (10 ng/ml) increased cell survival and prevented apoptotic DNA fragmentation and membrane PS exposure during OGD. DKK-1 application abrogates the protective capacity of EPO during OGD (*$P < 0.01$ *vs*. untreated control; †$P < 0.01$ *vs*. OGD). Each data point represents the mean and SEM from 6 experiments (*$P < 0.01$ *vs*. untreated control; $^{\dagger}P < 0.01$ *vs*. OGD).

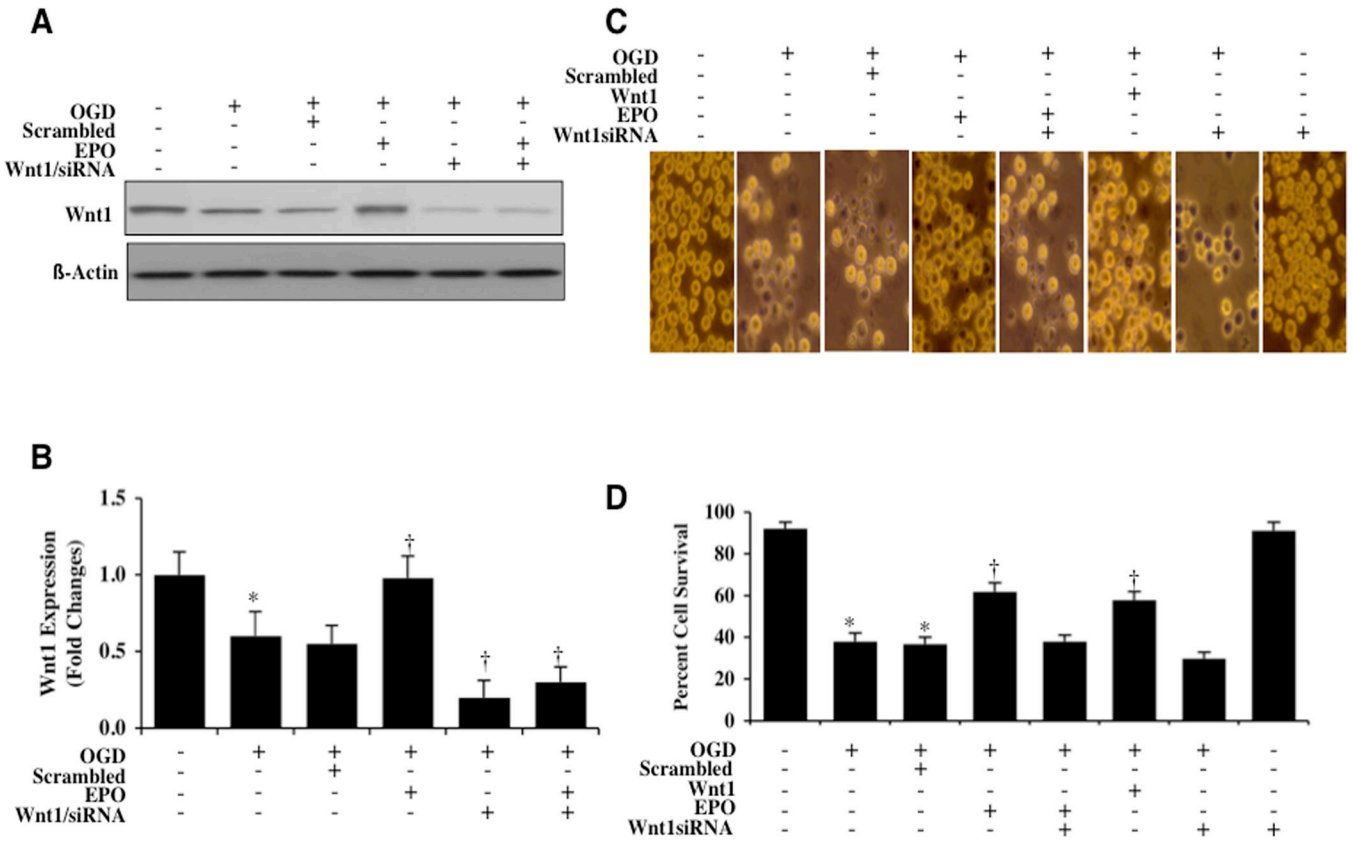

Fig. (5). Gene silencing of Wnt1 abrogates the protective capacity of EPO against OGD

(A and **B)** Gene silencing of *Wnt1* was performed with transfection of Wnt1 siRNA prior to OGD in microglia. Wnt1 expression was determined at 6 hours following a 6 hour period of OGD. Transfection with Wnt1 siRNA significantly reduced expression of Wnt1 following a 6 hour period of OGD or during EPO (10 ng/ml) application with OGD, but non-specific scrambled siRNA did not alter Wnt1 expression (**P* < 0.01 *vs*. OGD). In **B**, western band intensity was performed using the public domain NIH image program (http://rsb.info.nih.gov/nih-image). **(C)** Gene silencing of *Wnt1* was performed with transfection of Wnt1 siRNA prior to OGD in microglia and cell survival was determined by using trypan blue dye exclusion method 24 hours following a 6 hour period of OGD. Transfection with Wnt1 siRNA significantly increased cell staining during OGD and prevented protection by EPO (10 ng/ml) during OGD exposure resulting in increased trypan blue staining. Non-specific scrambled siRNA did not alter trypan blue staining during OGD. **(D)** Transfection with Wnt1 siRNA in microglia prior to OGD significantly reduced cell survival and blocked the ability of EPO (10 ng/ml) to protect microglia during OGD (**P* < 0.01 *vs*. untreated control; †*P*<0.01 *vs*. OGD). Each data point represents the mean and SEM from 6 experiments.

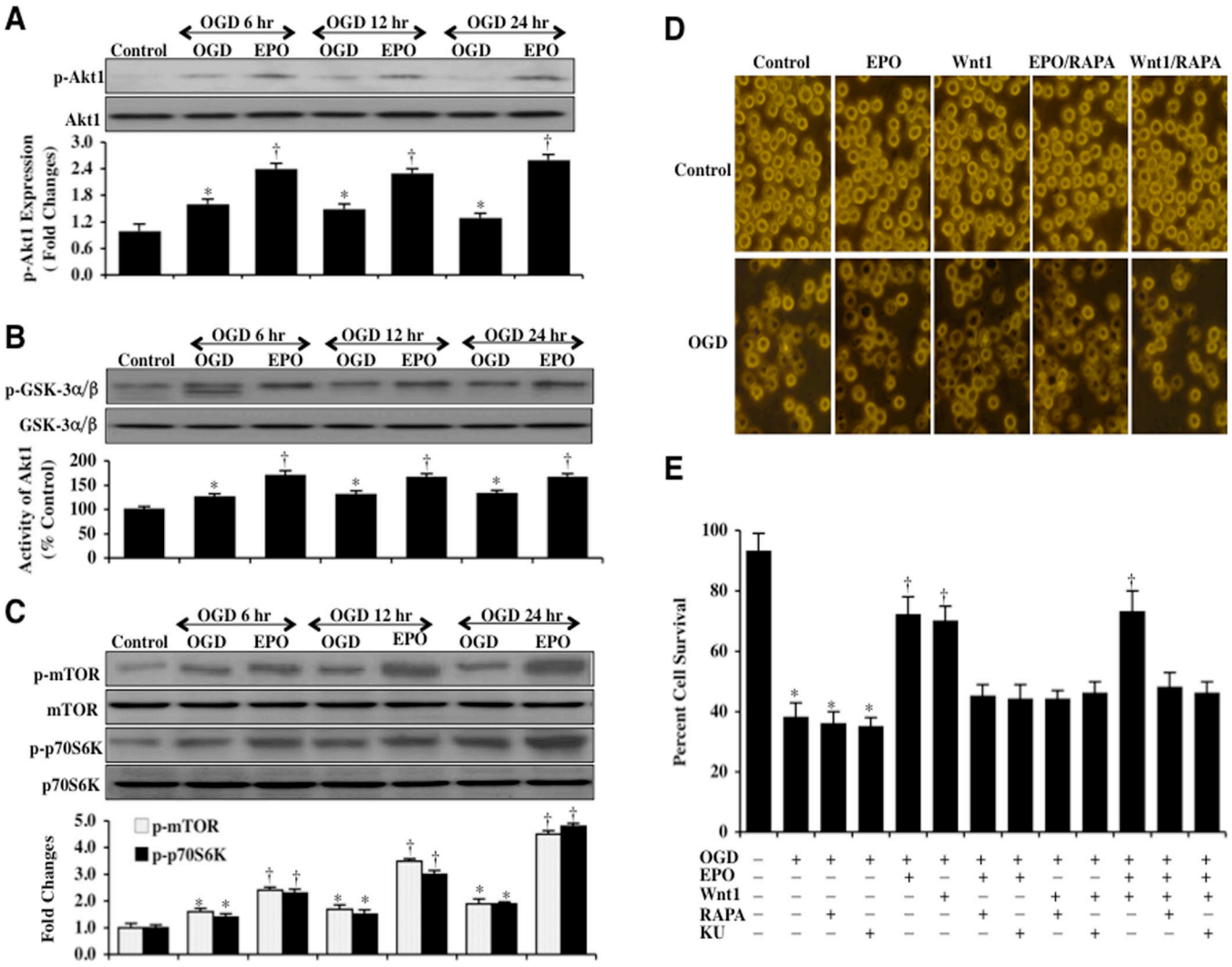

Fig. (6). EPO activates Akt1 and employs mTOR to protect microglia against OGD

(A) Microglial protein extracts (50 μg/lane) were immunoblotted with p-Akt1 (active form) at 6, 12, and 24 hours following a 6 hour period of OGD. OGD resulted in a slight increase in the expression of p-Akt1 but was progressively lost over a 24 hour period. EPO (10 ng/ml) with a 1 hour pretreatment significantly increased the expression of p-Akt1 (**P* <0.01 *vs*. Control; †*P*<0.01 *vs*. OGD of corresponding time point). In all cases, each data point represents the mean and SEM from 6 experiments. **(B)** The activity of Akt1 was determined by assessing the expression of p-GSK-3α/β after incubation of the substrate GSK-3 infusion protein with protein extracts from microglia following OGD. EPO (10 ng/ml) with 1 hour pretreatment significantly increased the activity of Akt1 over 24 hours following a 6 hour period of OGD (**P* <0.01 *vs*. Control; †*P*<0.01 *vs*. OGD of corresponding time point). In all cases, each data point represents the mean and SEM from 6 experiments. **(C)** Microglial protein extracts (50 μg/lane) were immunoblotted with phosphorylated (p)-mTOR (Ser^{2448}) and p-p70S6K (Th^{389}) antibodies at 6, 12, and 24 hours following a 6 hour period of OGD. OGD resulted in a slight increase in the expression of p-mTOR and p-p70S6K, but a 1 hour pretreatment with EPO (10 ng/ml) significantly increased and maintained the expression of p-mTOR and p-p70S6K over a 24 hour period following OGD exposure (**P* <0.01 *vs*. Control; †*P*<0.01 vs. OGD of corresponding time point). In all cases, each data point represents the mean and SEM from 3 experiments. **(D)** EPO (10 ng/ml) or Wnt1 (100 ng/ml)

was applied to microglial cultures 1 hour prior to a 6 hour period of OGD and cell survival was determined 24 hours following OGD by using trypan blue dye exclusion method. Representative pictures demonstrate that EPO or Wnt1 application significantly reduced trypan blue staining following OGD. Application of the mTOR specific inhibitor rapamycin (RAPA, 20 nM) combined with EPO (10 ng/ml) administration blocked protection by EPO resulting in an increased staining of trypan blue in microglia. **(E)** Quantification of data illustrates that microglial cell survival was significantly decreased 24 hours following a 6 hour period of OGD when compared to untreated microglial control cultures. In contrast, EPO (10 ng/ml), Wnt1 (100 ng/ml) or EPO combined with Wnt1 administration significantly increased cell survival to a similar level. In contrast, the mTOR specific inhibitors rapamycin (RAPA, 20 ng/ml) or Ku 0063794 (KU, 100 ng/ml) blocked protection by EPO, Wnt1 or EPO combined with Wnt1 during OGD resulting in a decrease in microglial cell survival (*$P < 0.01$ *vs*. untreated control; †$P < 0.01$ *vs*. OGD). Each data point represents the mean and SEM from 6 experiments.

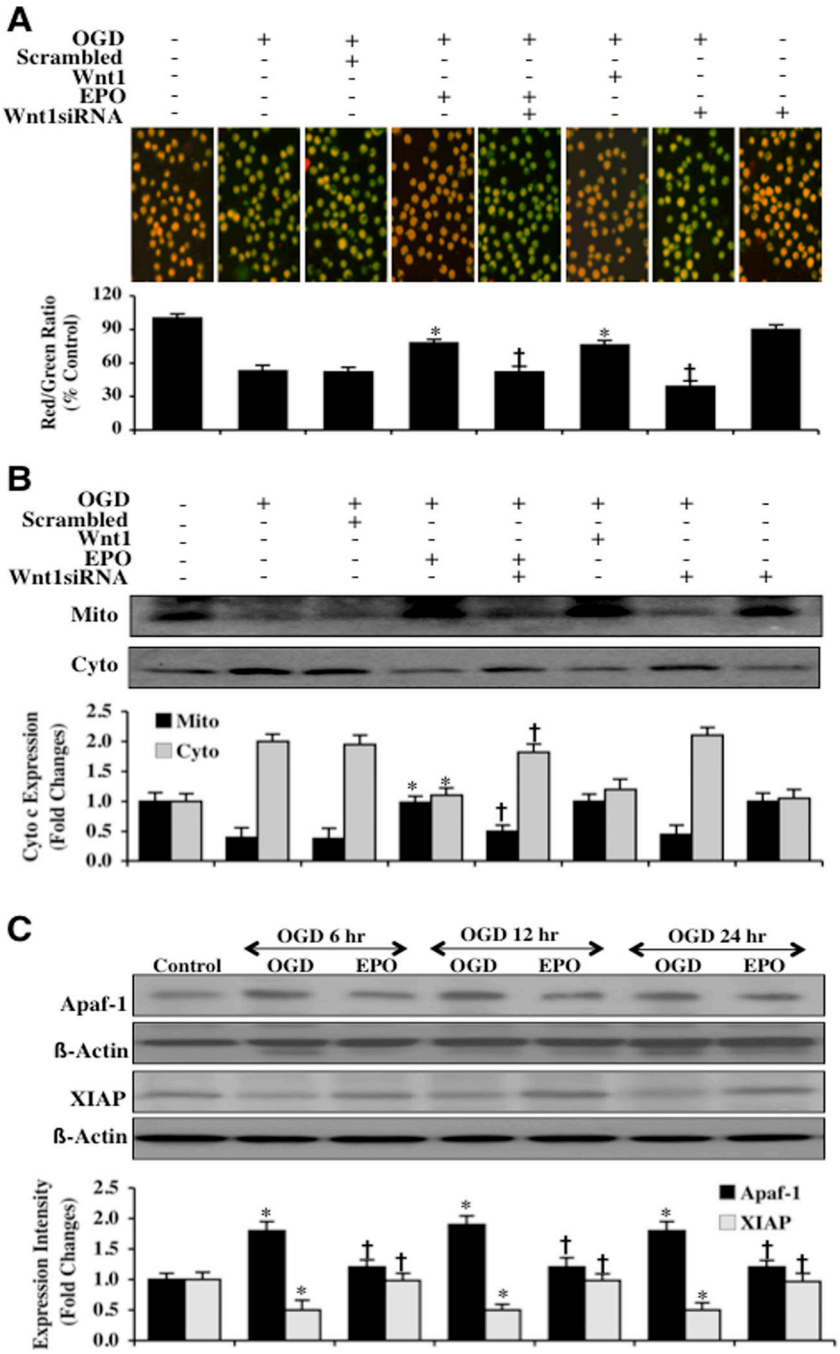

Fig. (7). EPO through Wnt1 inhibits mitochondrial depolarization and subsequent cytochrome c release and modulates the expression of Apaf-1 and XIAP

(**A**) OGD produced a significant decrease in the red/green fluorescence intensity ratio of mitochondria using a cationic membrane potential indicator JC-1 within 6 hours when compared with untreated control cultures demonstrating that OGD results in mitochondrial membrane depolarization. EPO (10 ng/ml) or Wnt1 (100 ng/ml) application during OGD prevented mitochondrial depolarization and significantly increased the red/green fluorescence intensity of mitochondria in microglia. In contrast, inhibition of Wnt1 with transfection of Wnt1 siRNA (siRNA) increased mitochondrial membrane depolarization to a greater degree than OGD alone and blocked the ability of EPO to prevent mitochondrial

depolarization during OGD. The relative ratio of red/green fluorescent intensity of mitochondrial staining was measured in 6 independent experiments with analysis performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image) (untreated microglia = Control, **P*<0.01 *vs*. OGD; †*P* <0.01 *vs*. EPO/OGD). (**B**) Equal amounts of mitochondrial (Mito) or cytosol (Cyto) protein extracts (50 μg/lane) were immunoblotted demonstrating that EPO or Wnt1 administration significantly prevented cytochrome c release from mitochondria during OGD (**P*<0.01 *vs*. OGD; †*P* <0.01 *vs*. EPO/OGD). Transfection with Wnt1 siRNA blocked the ability of EPO to prevent mitochondrial release of cytochrome c. Non-specific scrambled siRNA did not affect mitochondrial depolarization. Each data point represents the mean and SEM from 6 experiments. (**C**) Microglial protein extracts (50 μg/lane) were immunoblotted with Apaf-1 and XIAP antibodies 6, 12, and 24 hours following OGD. OGD significantly increased Apaf-1 and significantly decreased XIAP expression. In contrast, EPO (10 ng/ml) application decreased Apaf-1 and increased XIAP expression following OGD (**P* <0.01 *vs*. Control; †*P*<0.01 *vs*. OGD). In all cases, each data point represents the mean and SEM from 3 experiments.

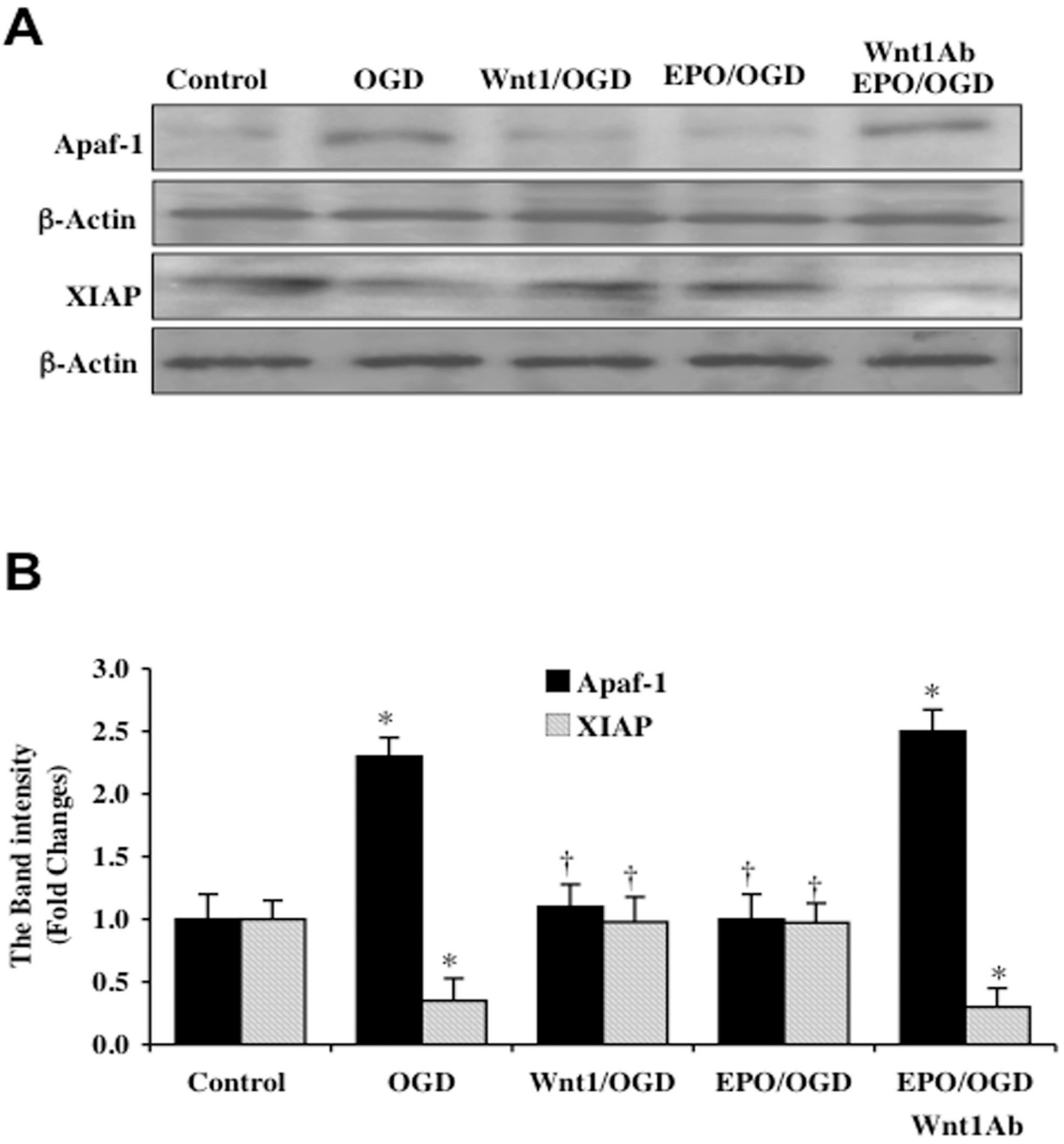

Fig. (8). EPO requires Wnt1 to govern expression of Apaf-1 and XIAP in microglia during OGD

Microglial protein extracts (50 μg/lane) were immunoblotted with Apaf-1 and XIAP antibodies 6 hours following a 6 hour period of OGD exposure. **(A** and **B)** A representative Western blot demonstrates that OGD exposure significantly increased Apaf-1 and significantly decreased XIAP expression. Application of EPO (10 ng/ml) 1 hour prior to OGD significantly decreased Apaf-1 and significantly increased XIAP expression following OGD. In addition, Wnt1 is necessary for EPO to control Apaf-1 and XIAP expression since application of Wnt1 Ab (1 μg/ml) with EPO blocks the ability of EPO during OGD exposure to decrease Apaf-1 expression (Figs. 8**A** and 8**B**) and increase XIAP expression. In **B,** quantification of western band intensity was performed using the public domain NIH

Image program (http://rsb.info.nih.gov/nih-image) (*P <0.01 *vs*. Control; $^{\dagger}P$<0.01 *vs*. OGD). In all cases, each data point represents the mean and SEM from 3 experiments.