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FoxO3a Governs Early Microglial Proliferation and Employs Mitochondrial Depolarization with Caspase 3, 8, and 9 Cleavage During Oxidant Induced Apoptosis

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Abstract

Microglia of the central nervous system have a dual role in the ability to influence the survival of neighboring cells. During inflammatory cell activation, microglia can lead to the disposal of toxic cellular products and permit tissue regeneration, but microglia also may lead to cellular destruction with phagocytic removal. For these reasons, it is essential to elucidate not only the underlying pathways that control microglial activation and proliferation, but also the factors that determine microglial survival. In this regard, we investigated in the EOC 2 microglial cell line with an oxygen-glucose deprivation (OGD) injury model of oxidative stress the role of the "O" class forkhead transcription factor FoxO3a that in some scenarios is closely linked to immune system function. We demonstrate that FoxO3a is a necessary element in the control of early and late apoptotic injury programs that involve membrane phosphatidylserine externalization and nuclear DNA degradation, since transient knockdown of *FoxO3a* in microglia preserves cellular survival 24 hours following OGD exposure. However, prior to the onset of apoptotic injury, FoxO3a facilitates the activation and proliferation of microglia as early as 3 hours following OGD exposure that occurs in conjunction with the trafficking of the unphosphorylated and active post-translational form of FoxO3a from the cytoplasm to the cell nucleus. FoxO3a also can modulate apoptotic mitochondrial signal transduction pathways in microglia, since transient knockdown of *FoxO3a* prevents mitochondrial membrane depolarization as well as the release of cytochrome c during OGD. Control of this apoptotic cascade also extends to progressive caspase activation as early as 1 hour following OGD exposure. The presence of FoxO3a is necessary for the expression of cleaved (active) caspase 3, 8, and 9, since loss of FoxO3a abrogates the induction of caspase activity. Interestingly, elimination of FoxO3a reduced caspase 9 activity to a lesser extent than that noted with caspase 3 and 8 activities, suggesting that

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FoxO3a in relation to caspase 9 may be more reliant upon other signal transduction pathways potentially independent from caspase 3 and 8.

Keywords

Apoptosis; bromodeoxyuridine; caspases; cytochrome c; forkhead transcription factors; FoxO3a; immune system; inflammation; microglia; mitochondria; oxidative stress; proliferating cell nuclear antigen

INTRODUCTION

Oxidative stress has a vital role in the pathology of multiple disease processes that can involve metabolic disorders (Barbosa *et al.*, 2008; Duarte *et al.*, 2008; Gossai and Lau-Cam, 2009; Guarnieri *et al.*, 2009; Hao *et al.*, 2009; Maiese, 2008a; Maiese, 2009b; Ruf *et al.*, 2009; Szabo, 2009; Wu *et al.*, 2009), cognitive dysfunction (Erol, 2009; Newman *et al.*, 2007; Power *et al.*, 2008; Toledano *et al.*, 2008), ischemic injury (Park *et al.*, 2009; Thomas *et al.*, 2008; Zhou *et al.*, 2009), cardiac, lung, and liver disease [109, 110, 111], psychiatric disorders [112, 113, 114], seizures (Lehtinen *et al.*, 2009; Sales Santos *et al.*, 2009), drug toxicity (Lu *et al.*, 2009; North *et al.*, 2003; Rosa *et al.*, 2007), and infertility [115, 116, 117]. Closely tied to oxidant cell injury is the process of apoptosis. Apoptosis consists of the destruction of genomic DNA (Maiese *et al.*, 1999; Maiese and Vincent, 2000a, b) as a later event during apoptotic injury (Dombroski *et al.*, 2000; Jessel *et al.*, 2002; Kang *et al.*, 2003b; Maiese and Vincent, 2000b) and the early externalization of membrane phosphatidylserine (PS) residues (Chong *et al.*, 2005b; Maiese *et al.*, 2008f). The loss of membrane phospholipid asymmetry leads to the exposure of membrane PS residues on the cell surface and the activation and proliferation of inflammatory microglial cells to target cells for phagocytosis (Chong *et al.*, 2003c; Kang *et al.*, 2003a, b; Maiese and Chong, 2003; Mallat *et al.*, 2005).

Interestingly, in many instances, the ultimate outcome of cells and their survival may be controlled by neighboring inflammatory cells such as microglia (Gilfillan and Rivera, 2009; Maiese *et al.*, 2009c). For example, during neurodegenerative disorders that can involve the loss of cognition and activation of microglial cells, phagocytic removal of both neurons and vascular cells can ensue (Chong *et al.*, 2007a; Maiese *et al.*, 2008d; Maiese *et al.*, 2005b). Within these periods of inflammatory cell activation, microglia rely upon their own intrinsic cytoprotective pathways (Chong *et al.*, 2007b; Li *et al.*, 2006b) to proliferate and remove cells that are no longer functional (Li *et al.*, 2005; Mallat *et al.*, 2005). Although microglia can be beneficial during periods of activation and proliferation to remove toxic cell products (Geijtenbeek and Gringhuis, 2009; Salminen and Kaarniranta, 2009) and allow for tissue regeneration (Chong *et al.*, 2007b; Dringen, 2005), microglia also can generate reactive oxygen species to lead to additional cell and tissue injury (Bakshi *et al.*, 2008; Denes *et al.*, 2008; Maiese, 2008b, 2009a; Maiese *et al.*, 2008a; Maiese *et al.*, 2008b; Williams *et al.*, 2009; Zhao *et al.*, 2009). As a result, it becomes critical to understand the pathways that can control not only the activation and proliferation of inflammatory microglia, but also the mechanisms that can limit survival of microglia.

One novel pathway that may be a viable candidate to modulate microglial function and survival involves the family of forkhead transcription factors. In particular, forkhead transcription factors of the “O” class (FoxOs) have intricate relationships with several vital cellular functions, such as metabolism (Maiese *et al.*, 2007a, 2008c; Maiese *et al.*, 2008e, f; Maiese *et al.*, 2007c) and immune surveillance (Maiese *et al.*, 2007b, 2008c). FoxOs are expressed in the reproductive system, cardiac and skeletal muscle, lung, liver, pancreas, spleen, thymus, and the nervous system (Lappas *et al.*, 2009; Maiese *et al.*, 2008e, 2009c, d). Furthermore, FoxOs,

such as the family member FoxO3a, can determine cellular survival in systems that involve metabotropic glutamate receptors (Chong *et al.*, 2006b), neurotrophins (Zheng *et al.*, 2002), cancer (Fei *et al.*, 2009; Maiese *et al.*, 2008e, 2009c), and cytokine (Chong and Maiese, 2007). For modulation of the immune system, FoxO3a activation may be required during disorders such as systemic lupus erythematosus (Sela *et al.*, 2006), T cell hyperactivity (Lin *et al.*, 2004), and immune complex-mediated inflammatory arthritis (Jonsson *et al.*, 2005; Ludikhuize *et al.*, 2007).

Given the ability of FoxO3a to oversee immune system function and cellular survival in a number of scenarios, we investigated the role of FoxO3a to modulate cerebral microglial activation and proliferation as well as intrinsic cellular pathways that are tied to apoptotic injury during oxidative stress. Here we show in the EOC 2 microglial cell line with an oxygen-glucose deprivation (OGD) injury model and transient gene knockdown of *FoxO3a* that early microglial activation and proliferation during oxidant stress induction depends upon FoxO3a. Furthermore, subsequent apoptotic injury programs with PS externalization and nuclear DNA degradation of microglia proceed through mitochondrial membrane depolarization, cytochrome c release, and activation of caspase 3, 8, and 9 that require the presence and intracellular trafficking of unphosphorylated (active) FoxO3a.

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% 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—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.

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 (Chong *et al.*, 2007b; Chong and Maiese, 2007; Kang *et al.*, 2003a, b). 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 (Pro-mega, 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.*, 2004a; 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.

Assessment of Microglial Activation and Proliferation

Proliferating cell nuclear antigen (PCNA) expression for microglial activation (Williams *et al.*, 2002) and bromideoxyuridine (BrdU) uptake for microglial proliferation (Martinez-Contreras *et al.*, 2002) was performed with anti-mouse monoclonal antibody PCNA (1:1000) or BrdU (1:6000) (Sigma, St Louis, MO) conjugated with biotinylated anti-mouse IgG (1:100) and visualized through fluorescein avidin (1:100) for PCNA and Texas Red streptavidin (Vector laboratories, Burlingame, CA) for BrdU. BrdU (10 μM) and fluorodexyuridine (1μM) (Sigma, St. Louis, MO) were applied 1 hour prior to the time of fixation.

Expression of Phosphorylated FoxO3a, total FoxO3a, and Active Caspase 3, 8, and 9

Cells were homogenized and each sample (50 μg $lane^{-1}$) was subjected to SDS-polyacrylamide gel electrophoresis (7.5% FoxO3a; 12.5% caspase 3, 8, and 9). After transfer, the membranes were incubated with a rabbit polyclonal antibody against a rabbit antibody against phospho-FoxO3a (1:1000) (p-FoxO3a, Ser^{253}, Cell Signaling, Beverly, MA), a rabbit antibody against total FoxO3a, and a rabbit antibody against cleaved (active) caspase 9 (37 kDa) (1:1000), a rabbit antibody against cleaved (active) caspase 8 (18 kDa) (1:1000), or a rabbit antibody against cleaved (active) caspase 3 (17 kDa) (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 (1:2000, 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/).

Transient Gene Knockdown of *FoxO3a* with Small Interfering RNA (siRNA)

To silence FoxO3a gene expression, the following sequences were synthesized (Ambion, Austin, Texas): the FoxO3a target sequence 5'- AAATCTAACTCATCTGCAA GT -3', the siRNA sense strand 5'-AUCUAACUCAUCUG CAAGUUU -3', and the antisense strand 5'-ACUUGCAG AUG AGUUAGAUUU -3'. Transfection of siRNA duplexes was performed with Lipofectamine 2000 reagent according to manufacturer guidelines (Invitrogen, Carlsbad, CA). Experimental assays were performed 72 hours post-transfection. For each siRNA assay, positive controls contain multiple siRNAs including the target siRNA and negative controls are absent of the target siRNA.

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. 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.

Preparation of Mitochondria for the Analysis of Cytochrome c Release

Per our prior protocols (Chong *et al.*, 2005a; Chong *et al.*, 2002a, 2003a, b, 2004a; Chong *et al.*, 2005f; Chong *et al.*, 2002b; Kang *et al.*, 2003a, b), 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 phenyl-methylsulfonylfluoride) 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.

Immunocytochemistry for FoxO3a and Caspase 3

For immunocytochemical staining of FoxO3a and cleaved caspase 3 (active form), microglial cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. Cells were then incubated with rabbit anti-FoxO3a (1:100, Cell Signaling Technology, Beverly, MA) or rabbit anti-cleaved caspase 3 (1:200, 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, then stained with DAPI (Sigma, St. Louis, MO) for nuclear identification. FoxO3a and caspase 3 proteins was imaged with fluorescence at the wavelengths of 565 nm (red) and 400 nm (DAPI nuclear staining).

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

Oxygen-Glucose Deprivation (OGD) Results in Cell Injury to Microglia

In order to examine the effect of OGD on microglial viability, we determined microglial survival 24 hours following OGD that was applied for a 6 hour period. As shown in Fig. (**1A**), representative pictures indicate that OGD lead to trypan blue staining in microglia, but no significant staining was found in control cells. In Fig. (**1B**), the quantification of data demonstrated that microglial survival was significantly reduced (37 ± 5%) 24 hours following a 6 hour period of OGD when compared to untreated control microglia (91 ± 4%, $p<0.01$). 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.

Early Phosphatidylserine (PS) Exposure and Subsequent Nuclear DNA Degradation Occurs During OGD Exposure

Oxidative stress, such as during OGD, leads to the destruction of cells through both apoptotic (Chong *et al.*, 2006a; De Felice *et al.*, 2007; Lin and Maiese, 2001) and autophagic pathways (Lee *et al.*, 2009). Furthermore, apoptosis is a dynamic process that consists of both the early exposure of membrane phosphatidylserine (PS) residues and the later destruction of genomic DNA (Chong *et al.*, 2005b; Maiese *et al.*, 2008f). Following a 6 hour period of OGD, apoptotic DNA fragmentation was determined by TUNEL and cell membrane PS exposure was assessed by annexin V 24 hours later. In Fig. (**1C**), untreated control cells were without DNA fragmentation or PS externalization. In cells exposed to OGD, significant DNA fragmentation

(TUNEL) and membrane PS exposure (annexin V) was present. In Fig. (**1D**), OGD lead to a significant increase in percent DNA fragmentation (59 ± 5%) and membrane PS exposure (61 ± 4%) in microglia 24 hours after OGD compared to untreated control cultures for DNA (8 ± 2%) and for PS (11 ± 4%) respectively.

OGD Leads to Activation and Proliferation of Microglia

Externalization of membrane PS residues is an early event during cell apoptosis (Maiese *et al.*, 2000; Mari *et al.*, 2004) that can become a signal for the phagocytosis of cells (Chong *et al.*, 2005a; Li *et al.*, 2006b; Lin and Maiese, 2001). The subsequent loss of membrane phospholipid asymmetry leads to the exposure of membrane PS residues on the cell surface and assists microglia to target cells for phagocytosis (Chong *et al.*, 2003c; Kang *et al.*, 2003a, b; Maiese and Chong, 2003; Mallat *et al.*, 2005). We therefore examined whether OGD results in the activation and proliferation of microglia given that OGD can lead to membrane PS exposure in microglia (Fig. **1**). In Fig. (**2A**), representative microglial cultures illustrate a significant increase in microglial activation and proliferation following OGD at 3 hours and 6 hours as evidenced by increased PCNA expression and BrdU uptake. Untreated control cells are without significant PCNA expression or BrdU uptake. In Fig. (**2B**), quantification of PCNA labeling demonstrates significant expression in PCNA at 3 hours (38 ± 8%, $p<0.01$) and 6 hours (58 ± 5%, $p<0.01$) compared to control microglia (18 ± 5%). Similarly, BrdU uptake was significantly increased to 42 ± 6% at 3 hours and 61 ± 5% at 6 hours following OGD compared to untreated control cells (26 ± 4%).

During OGD, Phosphorylation of FoxO3a is Reduced to Allow for Nuclear Translocation

Loss of post-translational phosphorylation of FoxO proteins can prevent association of FoxOs with 14-3-3 proteins (Chong and Maiese, 2007; Maiese *et al.*, 2005b) and allow FoxOs to translocate to the cell nucleus to initiate a “pro-apoptotic” program (Maiese *et al.*, 2009a; Maiese *et al.*, 2009c, d; Shang *et al.*, 2009). We therefore examined the role of OGD during this process. Since unphosphorylated FoxO3a can translocate to the cell nucleus (Chong and Maiese, 2007; Maiese *et al.*, 2008c; Maiese *et al.*, 2008e, 2009d), we initially investigated the phosphorylation of FoxO3a 6 hours following OGD by using western blot analysis. Western blot assay was performed for phosphorylated FoxO3a (p-FoxO3a) at the preferential phosphorylation site for protein kinase B (Akt) of Ser^{253} as well as for the expression of total FoxO3a at 6 hours following OGD (Figs. **3A, 3B**). After 6 hours following OGD, expression of phosphorylated (inactive) p-FoxO3a was significantly decreased but expression of total FoxO3a remained unchanged, suggesting that the unphosphorylated and active FoxO3a form was present and that the total FoxO3a protein was not degraded (Figs. **3A, 3B**).

These observations were supported by our studies that show that the active FoxO3a transcription factor translocates from the cell cytosol to the nucleus during this 6 hour period following OGD. In microglia, we next performed immunofluorescent staining for FoxO3a and DAPI nuclear staining to follow the subcellular translocation of FoxO3a 6 hours following OGD (Figs. **3C, 3D**). Significant immunofluorescent staining for FoxO3a in the nucleus of microglia is present during OGD. This is evident by the inability to detect significant DAPI nuclear staining (blue in color) in cells during merged OGD images since prominent FoxO3a staining is present in the nucleus (Figs. **3C, 3D**). In contrast, in untreated control cells, FoxO3a is maintained in the cytoplasm with minimal nuclear staining as shown with DAPI staining (white in color) in the nucleus in cells in merged images. In Fig. (**3D**), data analysis illustrated that microglial nuclear staining area is significantly increased (189 ± 5%) 24 hours following a 6 hour period after OGD when compared to untreated control cells (107 ± 7%).

Transient Gene Knockdown of *FoxO3a* Prevents Microglial Injury During OGD

Microglia were transfected with FoxO3a siRNA and the expression of total FoxO3a protein was documented with Western blot analysis (Figs. **4A, 4B**). Transient gene knockdown of *FoxO3a* in either untreated control cells or in cells exposed to OGD alone resulted in markedly reduced or absent expression of total FoxO3a 6 hours after OGD (Figs. **4A, 4B**). As a control, non-specific scrambled FoxO3a siRNA did not alter total FoxO3a expression in untreated control cells or cells exposed to OGD, illustrating the specificity for FoxO3a siRNA to block protein expression of total FoxO3a (Figs. **4A, 4B**). Representative figures illustrate significant trypan blue staining in microglial cells 24 hours after OGD administration alone or with OGD during scrambled (non-specific) siRNA (Fig. **4C**). In contrast, markedly reduced trypan blue uptake is present in microglia following OGD with FoxO3a siRNA transfection for 3 days (Fig. **4C**), demonstrating that the presence of FoxO3a contributes to microglial injury during OGD. On further analysis in Fig. (**4D**), percent microglial survival was increased from 37 ± 5% during OGD administration alone to 59 ± 4% ($p<0.01$) with OGD and FoxO3a siRNA 24 hours after OGD administration. Transfection with scrambled siRNA did not prevent microglial injury during OGD.

Absence of FoxO3a Protein can Limit Apoptotic DNA Fragmentation and Phosphatidylserine (PS) Exposure and Block the Early Activation and Proliferation of Microglia

We further examined the role of FoxO3a during OGD with apoptotic genomic DNA fragmentation and membrane PS exposure in microglia through TUNEL and annexin V analysis. In Fig. (**5A**), representative figures show a significant decrease in DNA fragmentation and PS exposure during OGD in microglia transfected with FoxO3a siRNA. Transfection with non-specific scrambled siRNA did not prevent DNA fragmentation or PS exposure in microglial cells. Quantification of these observations illustrate that transfection of microglia with FoxO3a siRNA during OGD decreased DNA fragmentation from 59 ± 5% (OGD alone) to 35 ± 2% with FoxO3a siRNA and decreased apoptotic PS exposure from 62 ± 5% (OGD alone) to 38 ± 4% with FoxO3a siRNA (Fig. **5B**).

In Fig. (**6A**), representative microglial cells demonstrate a significant increase in the activation (PCNA expression) and proliferation (BrdU uptake) of microglia at 6 hours after OGD when compared to untreated control microglia. In contrast, microglial transfection with scrambled siRNA during OGD also led to a marked increase in PCNA expression and BrdU uptake during OGD. In contrast, the expression of PCNA and BrdU uptake was significantly reduced in cells with FoxO3a siRNA transfection. In Fig. (**6B**), transfection of microglia with FoxO3a siRNA that was assessed 6 hours following OGD decreased PCNA expression from 64 ± 5% (OGD alone) to 32 ± 5% during OGD with FoxO3a siRNA and decreased BrdU uptake from 68 ± 5% (OGD alone) to 37 ± 4% during OGD with FoxO3a siRNA. Non-specific scrambled siRNA transfection did not prevent the increase in PCNA expression or BrdU uptake during OGD, illustrating the ability of FoxO3a to lead to early activation and proliferation in microglia.

Transient Gene Knockdown of *FoxO3a* Blocks Mitochondrial Depolarization and the Release of Mitochondrial Cytochrome c

OGD yielded a significant decrease in the mitochondrial red/green fluorescence intensity ratio within 6 hours (27 ± 5%) with labeling of microglial mitochondria by the cationic membrane potential indicator JC-1 when compared to untreated control mitochondria (97 ± 4%) (Figs. **7A, 7B**), demonstrating that OGD produces mitochondrial membrane depolarization. Transfection of FoxO3a siRNA in microglia prior to OGD exposure significantly increased the red/green fluorescence intensity of the mitochondria (82 ± 5%), indicating that mitochondrial permeability transition pore membrane potential was markedly improved (Figs. **7A, 7B**). In contrast, non-specific scrambled siRNA during OGD did not prevent mitochondrial

membrane depolarization, suggesting that FoxO3a is necessary, at least in part, for OGD to lead to the depolarization of the mitochondrial membrane.

In regards to cytochrome c release, OGD within 6 hours resulted in a marked release of cytochrome c from the mitochondria to a 3.3 ± 0.3% fold increase when compared to untreated control mitochondria (Figs. **7C, 7D**) using western analysis. Non-specific scrambled siRNA did not alter this release of cytochrome c during OGD exposure, but transfection of mitochondria with FoxO3a siRNA prevented cytochrome c release to a similar degree that occurs with untreated control microglial mitochondria (Figs. **7C, 7D**).

Activities of Caspase 3, 8, and 9 are Controlled by FoxO3a During OGD

Caspases are a family of cysteine proteases that are synthesized as inactive zymogens and are proteolytically cleaved into subunits during loss of mitochondrial membrane permeability (Li *et al.*, 2006a; Maiese *et al.*, 2005a; Okouchi *et al.*, 2007). We therefore elected to investigate the ability of FoxO3a to modulate caspase 3, 8, and 9 activity. In Figs. (**8A, 8B**), cleaved caspase 3 immunocytochemistry reveals significant active caspase 3 (blue/red staining) within 6 hours following OGD exposure and during transfection of nonspecific scrambled siRNA. However, transfection of FoxO3a siRNA in microglia significantly blocks caspase 3 activity as evidenced by primarily blue immunocytochemical staining and by reducing the percentage of cleaved caspase 3 labeling to 8 ± 3% from 68 ± 4% in microglia exposed to OGD alone (Figs. **8A, 8B**). In addition, Figs. (**8C, 8D**) demonstrate that on western analysis expression of cleaved active caspase 3 is not present or at minimal levels similar to untreated controls during FoxO3a transfection at very early time periods of either 1 hour or 6 hours following OGD exposure. Almost paralleling the observations with active caspase 3 during FoxO3a transfection, expression of cleaved active caspase 8 (Figs. **9A, 9B**) and expression of cleaved active caspase 9 are elevated approximately 4 fold over untreated control microglia levels at 1 hour and 6 hours following OGD, but transfection with FoxO3a siRNA significantly blocks cleaved active caspase 8 activity (Figs. **9A, 9B**) and markedly reduces, although to a lesser extent, cleaved active caspase 9 activity (Figs. **9C, 9D**). Further supporting the ability of FoxO3a to lead to the activation of caspase 8 and caspase 9 are the observations that non-specific scrambled siRNA was ineffective in reducing caspase 8 or caspase 9 activity during OGD at 1 hour and at 6 hours (Figs. **9A, 9B, 9C, 9D**).

DISCUSSION

Oxidative stress and apoptotic injury involves several cell types that include neurons, endothelial cells, cardiomyocytes, and smooth muscle cells (Chong *et al.*, 2004a; Chong *et al.*, 2007b; Harris *et al.*, 2007; Kang *et al.*, 2003b; Karunakaran *et al.*, 2007; Verdaguer *et al.*, 2007). In addition, inflammatory cells of the brain are no exception to this list of cells affected by oxidative stress (Bureau *et al.*, 2008; Chong *et al.*, 2005a; Chong *et al.*, 2003b, 2004a; Chong *et al.*, 2007b; Chu *et al.*, 2008; Denes *et al.*, 2008; Li *et al.*, 2006b; Park *et al.*, 2009; Power *et al.*, 2008; Sanchez *et al.*, 2009; Shang *et al.*, 2009; Zhao *et al.*, 2009). We show that OGD exposure for a period of 6 hours results in a significantly reduced survival rate for microglia with DNA fragmentation and early apoptotic changes associated with membrane PS exposure over a 24 hour course. However, cell injury assessed by trypan blue exclusion is significantly limited in microglia following OGD exposure during transient gene knockdown of *FoxO3a*, illustrating that the presence of FoxO3a is a necessary component for microglial injury during OGD. Furthermore, a significant decrease in apoptotic DNA fragmentation and membrane PS exposure during OGD in microglia transfected with FoxO3a siRNA occurs. In studies with both cell survival and apoptotic DNA degradation and PS exposure, transfection with non-specific scrambled siRNA did not prevent injury in microglial cells, supporting the specificity of FoxO3a to control cell injury and apoptotic early and late programs in microglia.

Our studies are consistent with prior work that demonstrate FoxO3a must be present for oxidant stress – induced apoptosis (Nakamura and Sakamoto, 2007), that FoxO3a controls an apoptotic ligand activating a Fas-mediated death pathway in motoneurons (Barthelemy *et al.*, 2004), and that FoxO3a in conjunction with tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) and BH3-only proteins results in apoptotic injury in neuroblastoma cells (Obexer *et al.*, 2007). In addition, removal of FoxO expression during oxidative stress has been shown to be cytoprotective. Protein inhibition or gene knockdown of FoxO3a can reduce cerebral ischemia (Won *et al.*, 2006), mediate protection of metabotropic glutamate receptors during vascular injury (Chong *et al.*, 2006b), enhance pancreatic β-cell or neuronal survival through NAD^+ precursors during oxidative stress (Chong *et al.*, 2004b), provide trophic factor protection with erythropoietin (Chong and Maiese, 2007) and neurotrophins (Caporali *et al.*, 2008), and protect microglia against amyloid toxicity (Shang *et al.*, 2009).

However, prior to the onset of cell injury and apoptosis in microglia during oxidative stress, FoxO3a also controls a novel function of microglia. During initial OGD exposure within 3 and 6 hours, microglia have a significant increase in activation (PCNA expression) and proliferation (BrdU uptake) when compared to untreated control microglia. In contrast, the expression of PCNA and BrdU uptake in microglia was significantly reduced in cells with FoxO3a siRNA transfection while microglial transfection with scrambled siRNA during OGD resulted in a marked increase in PCNA expression and BrdU uptake during OGD. During these early time periods of 3 and 6 hours following OGD exposure, we also we investigated the phosphorylation of FoxO3a. After 6 hours following OGD, expression of phosphorylated (inactive) p-FoxO3a was significantly decreased but expression of total FoxO3a remained unchanged, suggesting that the unphosphorylated and active post-translational form of FoxO3a was most likely present and that the total FoxO3a protein was not destroyed. These observations were further supported by our work that demonstrates that the active FoxO3a transcription factor translocates from the cell cytosol to the nucleus during this same time period (within 6 hours following OGD). Post-translational modification of FoxO3a that yields an unphosphorylated (active) state allows FoxO3a to not associate with 14-3-3 proteins in the cytosol and to shuttle to the nucleus to allow for transcriptional activity (Chong and Maiese, 2007; Maiese *et al.*, 2009a; Maiese *et al.*, 2009b; Maiese *et al.*, 2009e).

The protection of mitochondrial function also may be an important factor for FoxO3a to regulate microglial cell survival. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) through the opening of the mitochondrial permeability transition pore and the release of cytochrome c represents a significant determinant for cell injury (Leuner *et al.*, 2007; Maiese and Chong, 2004) and the induction of apoptosis (Chong *et al.*, 2002a; Chong *et al.*, 2003d; Miki *et al.*, 2006). In HCT116 cells, FoxO3a has been shown to interact with the mitochondrial sirtuin SIRT3 (Jacobs *et al.*, 2008), suggesting that FoxO3a can control mitochondrial signal transduction pathways. In addition, FoxO3a has been shown to lead to cytochrome c release in neuroblastoma cells and neurons (Chong *et al.*, 2004b; Obexer *et al.*, 2007) and initiate caspase activation in microglial cells (Shang *et al.*, 2009). In our current studies, we show that OGD in microglia leads to mitochondrial membrane depolarization and cytochrome c release within 6 hours after OGD exposure. However, transient knockdown of *FoxO3a* in microglia prevents mitochondrial membrane depolarization as well as the release of cytochrome c during OGD, illustrating that FoxO3a is required, at least in part, for the initiation of mitochondrial pathways that can lead to apoptotic cell injury in microglia.

Since mitochondrial function is closely linked to caspase activity, we next investigated the role of specific caspases with FoxO3a. Although initiator caspases, such as caspase 8 and 9, are necessary to activate downstream effector caspases, it is the downstream effector caspases, such as caspase 3, that can directly lead to DNA destruction (Chong *et al.*, 2005d, e; Maiese *et al.*, 2005a; Maiese *et al.*, 2008f) and cellular membrane PS exposure (Chong *et al.*, 2003a;

Chong *et al.*, 2003d; Takahashi *et al.*, 1999). In addition, caspase 3 is tied to a unique regulatory mechanism that leads to proteolytic degradation of phosphorylated FoxO3a that potentially can enhance the vulnerability of cells to apoptotic injury (Charvet *et al.*, 2003). Prior work also has shown that FoxO3a activity promotes caspase-induced apoptotic death (Chong *et al.*, 2006b; Chong *et al.*, 2004b; Chong and Maiese, 2007; Obexer *et al.*, 2007), but inhibition of caspase 3 also can maintain the phosphorylated "inactive" state of FoxO3a to prevent cell injury (Chong *et al.*, 2006b; Chong *et al.*, 2004b; Chong and Maiese, 2007). Additional studies have shown that caspase 3 activity and cleavage is promoted during transfection of a triple mutant FoxO3a expression in which three phosphorylation sites have been altered to prevent inactivation of FoxO3a (Gomez-Gutierrez *et al.*, 2006). Furthermore, FoxO3a may control early activation and subsequent apoptotic injury in microglia during amyloid exposure through caspase 3 (Shang *et al.*, 2009).

We show that cleaved caspase 3 activity through immunocytochemistry is significantly increased within 6 hours after OGD. In contrast, transfection of FoxO3a siRNA in microglia significantly blocked caspase 3 activity. Furthermore, FoxO3a was able to lead to a rapid and marked increase in cleaved caspase 3 expression on western analysis with 1 and 6 hours following OGD, since loss of *FoxO3a* during transient gene knockdown abrogated increases in caspase 3 activity during OGD. Similar to our observations with caspase 3 during FoxO3a transfection, expression of cleaved active caspase 8 was increased almost 4 fold over untreated control microglia levels at 1 hour and 6 hours following OGD, but transient gene knockdown of *FoxO3a* also significantly prevented cleaved active caspase 8 activity.

Of interest is our observation that loss of FoxO3a reduced caspase 9 activity to a lesser extent than that noted with caspase 3 and 8 activities, suggesting that FoxO3a in relation to caspase 9 may be more reliant upon other signal transduction pathways potentially independent from caspase 3 and 8. One potential pathway that comes to mind is protein kinase B (Akt). Activation of Akt is usually cytoprotective, such as during cell proliferation (Gayer *et al.*, 2009), ischemia/stress (An *et al.*, 2008; Tsolakidou *et al.*, 2008), hypoxia (Chong *et al.*, 2002a), β-amyloid toxicity (Chong *et al.*, 2005c), cardiomyopathy (Kim *et al.*, 2008), cellular aging (Tajes *et al.*, 2009), neurodegeneration (Morissette *et al.*, 2008a; Morissette *et al.*, 2008b), and oxidative stress (Chong *et al.*, 2004a; Kang *et al.*, 2003a, b). In relation to the modulation of FoxO3a activity, Akt can prevent cellular apoptosis through the phosphorylation of FoxO3a (Maiese *et al.*, 2008e, 2009c) and maintain FoxO3a in the cytoplasm by association with 14-3-3 proteins (Chong and Maiese, 2007; Maiese *et al.*, 2005b). In addition, cytoprotection through Akt can involve the maintenance of mitochondrial membrane potential, prevention of cytochrome c release, and blockade of caspase activity including caspase 9 (Chong *et al.*, 2005a; Chong *et al.*, 2002a; Kang *et al.*, 2003a, b), raising the possibility that Akt may be more influential in limiting the effects of FoxO3a on caspase 9 activity during oxidative stress.

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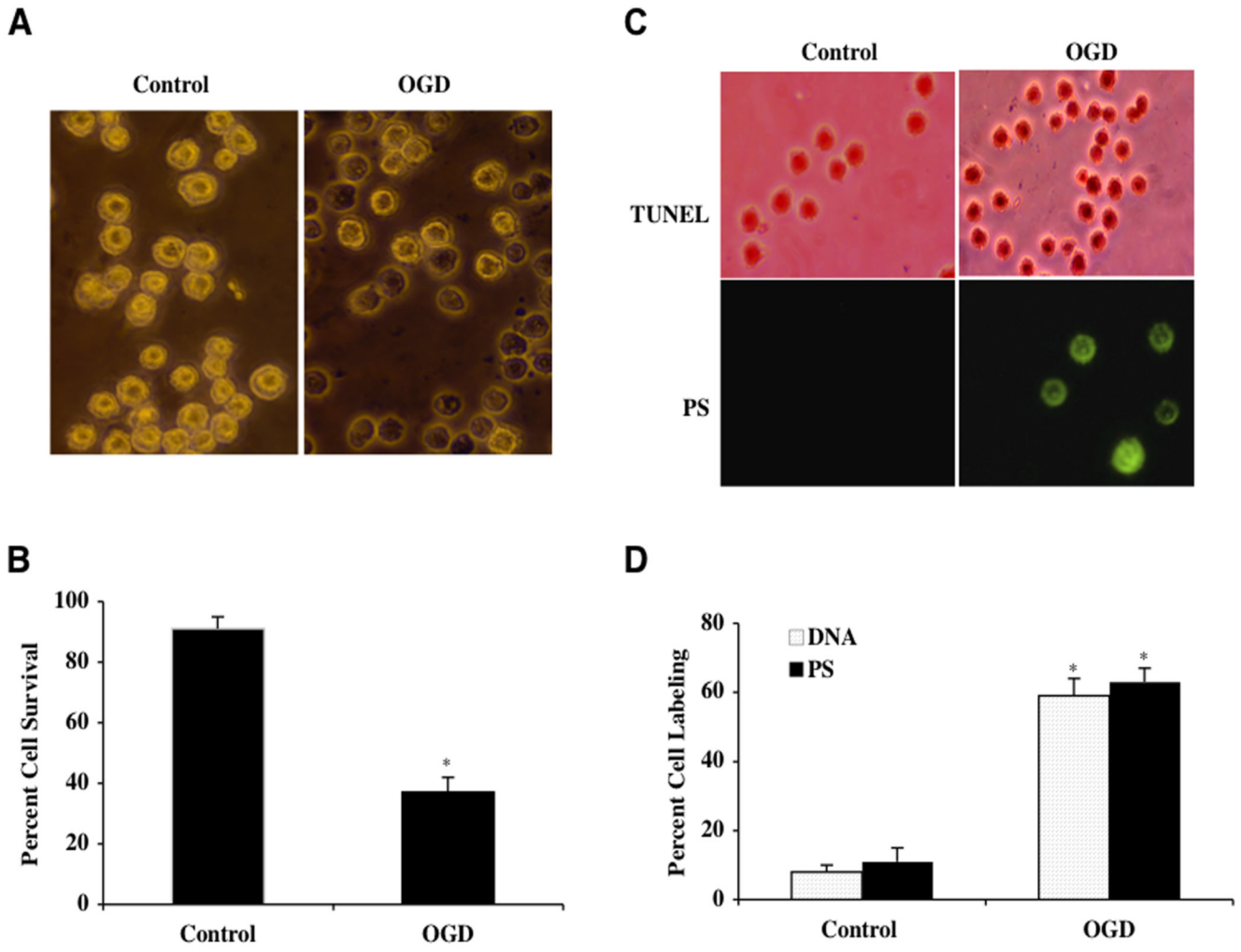

Fig. (1). Oxygen-glucose deprivation (OGD) results in microglial injury with early and late apoptotic damage

(A) Microglial cells were exposed to OGD and cell survival was determined 24 hours after OGD with the trypan blue exclusion method. Representative images show that OGD leads to significant trypan blue uptake. **(B)** Microglial survival was decreased with OGD exposure (**P*<0.01 *vs*. untreated control). Each data point represents the mean and SEM from 6 experiments. **(C)** Representative images show that OGD leads to apoptotic DNA fragmentation (dark nuclei) with TUNEL stain and membrane PS externalization (green fluorescence) with annexin V twenty-four hours following OGD. **(D)** OGD exposure significantly increased DNA fragmentation and PS exposure in microglia (**P*<0.01 *vs*. untreated control). Each data point represents the mean and SEM from 6 experiments.

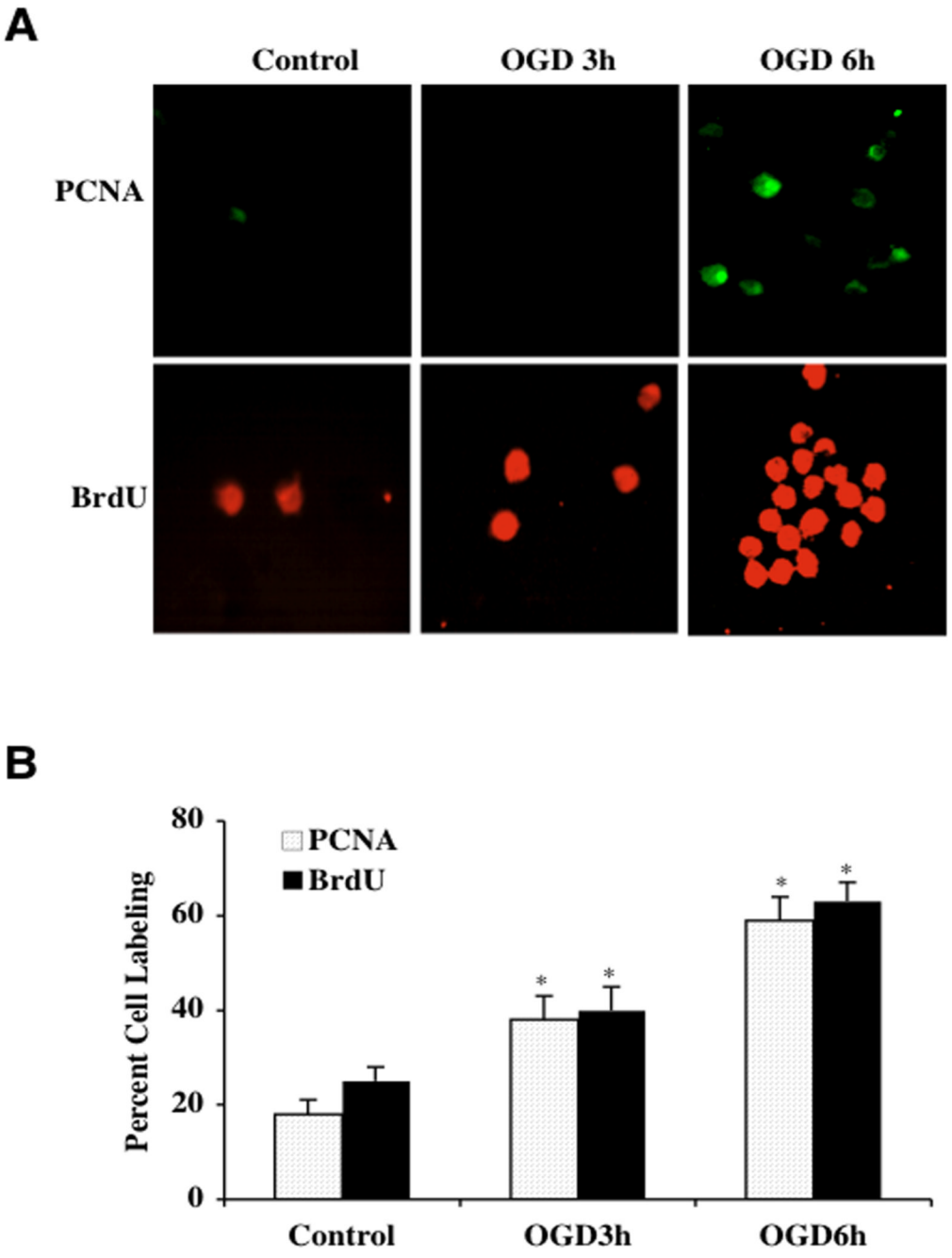

Fig. (2). Early microglial activation and proliferation is present with OGD

(A) Representative images and quantitative analysis illustrate that PCNA and BrdU expression is significantly and rapidly increased in microglia at 3 hours and 6 hours after OGD (**P*<0.01 *vs*. control). **(B)** Quantification of data demonstrate that PCNA and BrdU were significantly increased following OGD (*p<0.01 *vs*. control). In all cases, control = untreated cells. Each data point represents the mean and SEM from 6 experiments.

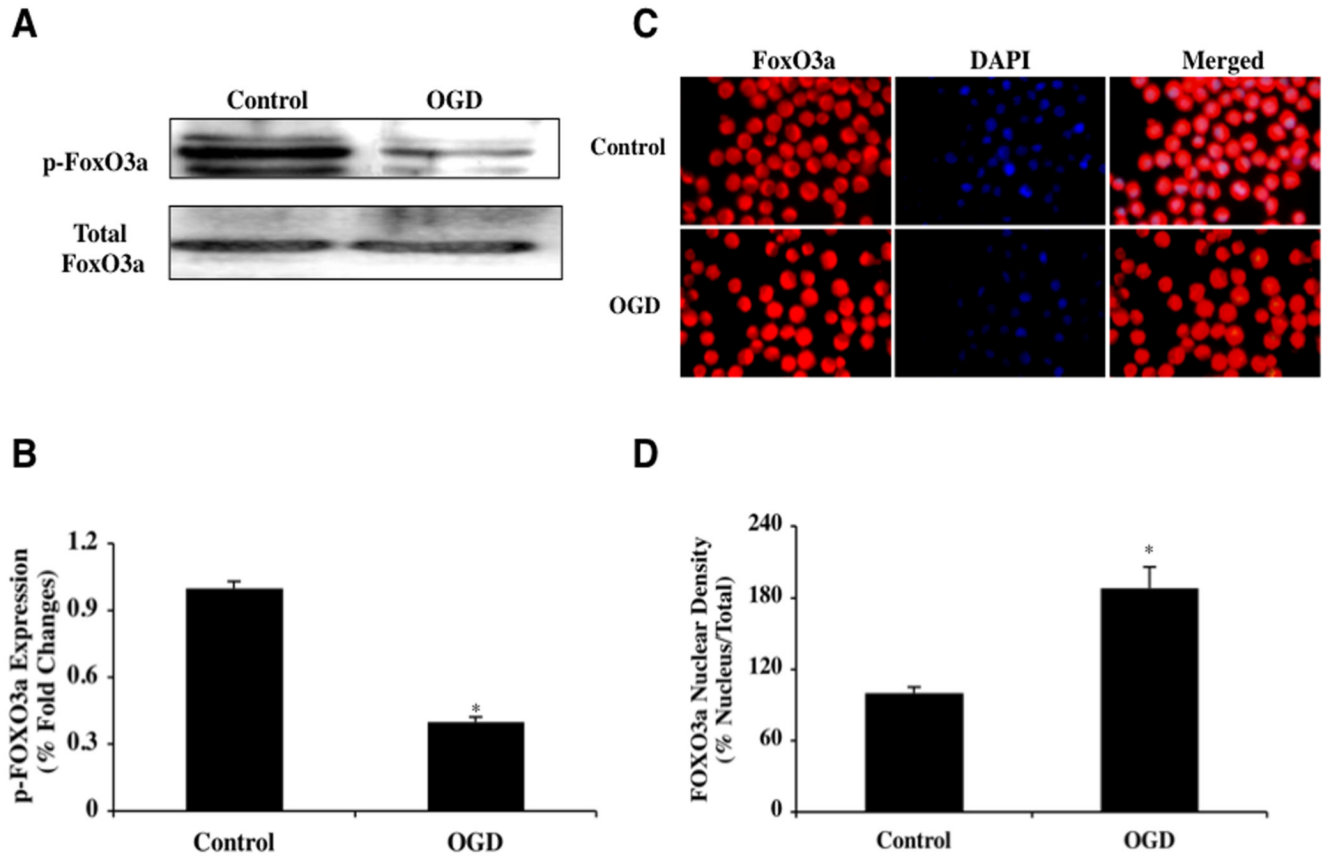

Fig. (3). OGD leads to loss of phosphorylation of FoxO3a with subsequent subcellular trafficking to the nucleus

In **A** and **B**, microglial protein extracts (50 μ/lane) were immunoblotted with anti-phosphorylated-FoxO3a (p-FoxO3a, Ser^{253}) or anti-total FoxO3a at 6 hours following OGD. Phosphorylated (inactive) FoxO3a (p-FoxO3a) expression is significantly decreased 6 hours following OGD but total FoxO3a is not affected (**P*<0.01 *vs*. control). In **C** and **D**, microglia were imaged 6 hours following OGD with immunofluorescent staining for FoxO3a (Texas-red streptavidin). Nuclei of microglia were counterstained with DAPI. In merged images, untreated control microglia have readily visible nuclei (dark white in color) that illustrate absence of FoxO3a in the nucleus. In contrast, merged images after OGD have completely red cytoplasm and nucleus with minimal visibility of the nucleus with DAPI illustrating translocation of FoxO3a to the nucleus. Quantification of the intensity of FoxO3a nuclear staining was performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image) (**P*<0.01 *vs*. control). Control = untreated microglia.

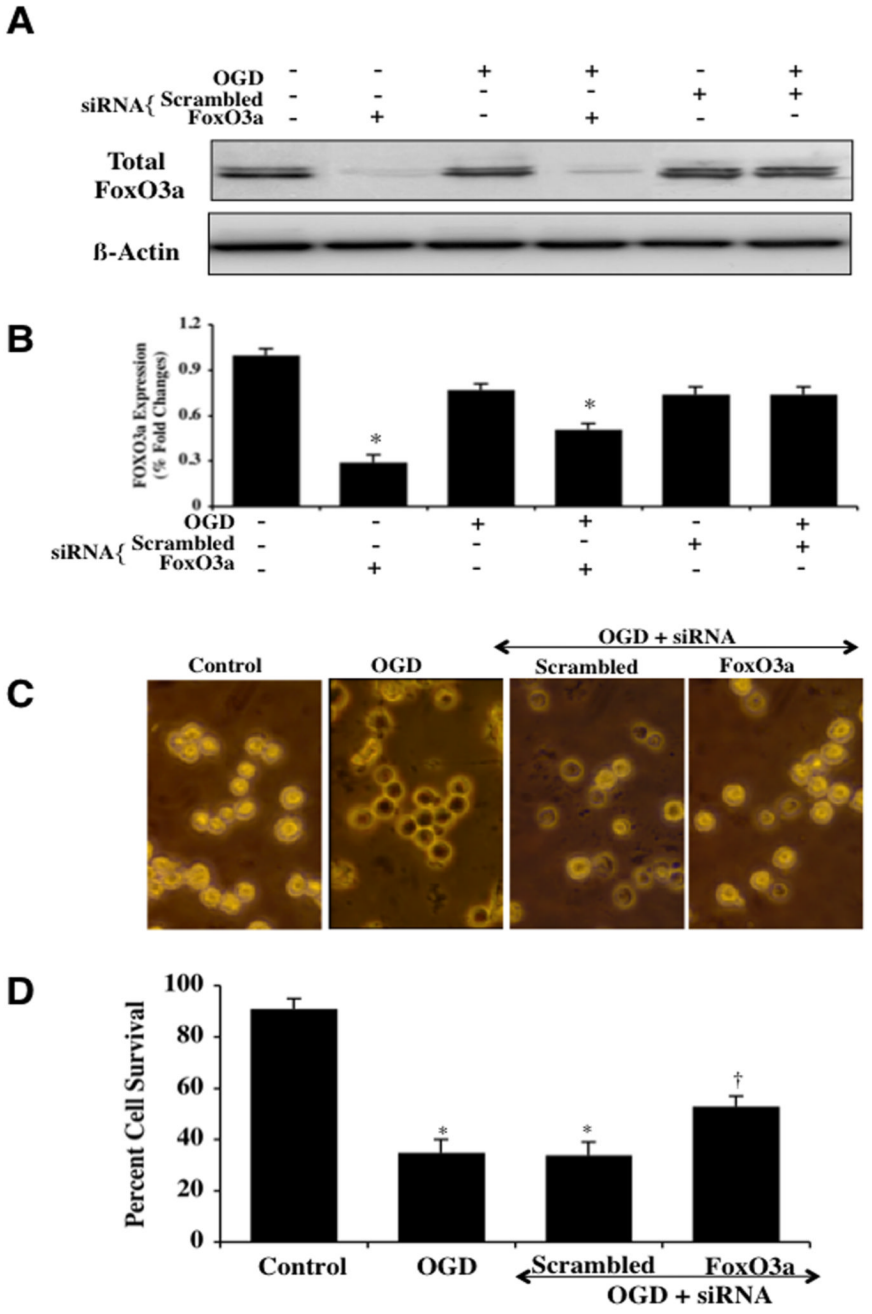

Fig. (4). Transient gene knockdown of *FoxO3a* increases microglial survival during OGD

In **A** and **B**, microglial protein extracts (50 μ/lane) were immunoblotted with anti-phosphorylated-FoxO3a (p-FoxO3a, Ser^{253}) or anti-total FoxO3a at 6 hours following OGD. Transient gene knockdown of *FoxO3a* was performed with transfection of FoxO3a siRNA (siRNA). FoxO3a siRNA significantly reduced expression of total FoxO3a alone or following OGD but non-specific scrambled siRNA did not alter total FoxO3a expression (**P*<l0.01 *vs*. control). In **C** and **D**, gene knockdown of *FoxO3a* with FoxO3a siRNA (siRNA) significantly increased microglial survival and decreased microglial membrane injury assessed by trypan blue staining 24 hours after OGD (**P*<0.01 *vs*. Control; †*P* <0.01 *vs*. OGD). FoxO3a siRNA alone was not toxic and non-specific scrambled siRNA did not protect cells during OGD

(*P<0.01 *vs*. untreated control cells). In all cases, each data point represents the mean and SEM from 6 experiments.

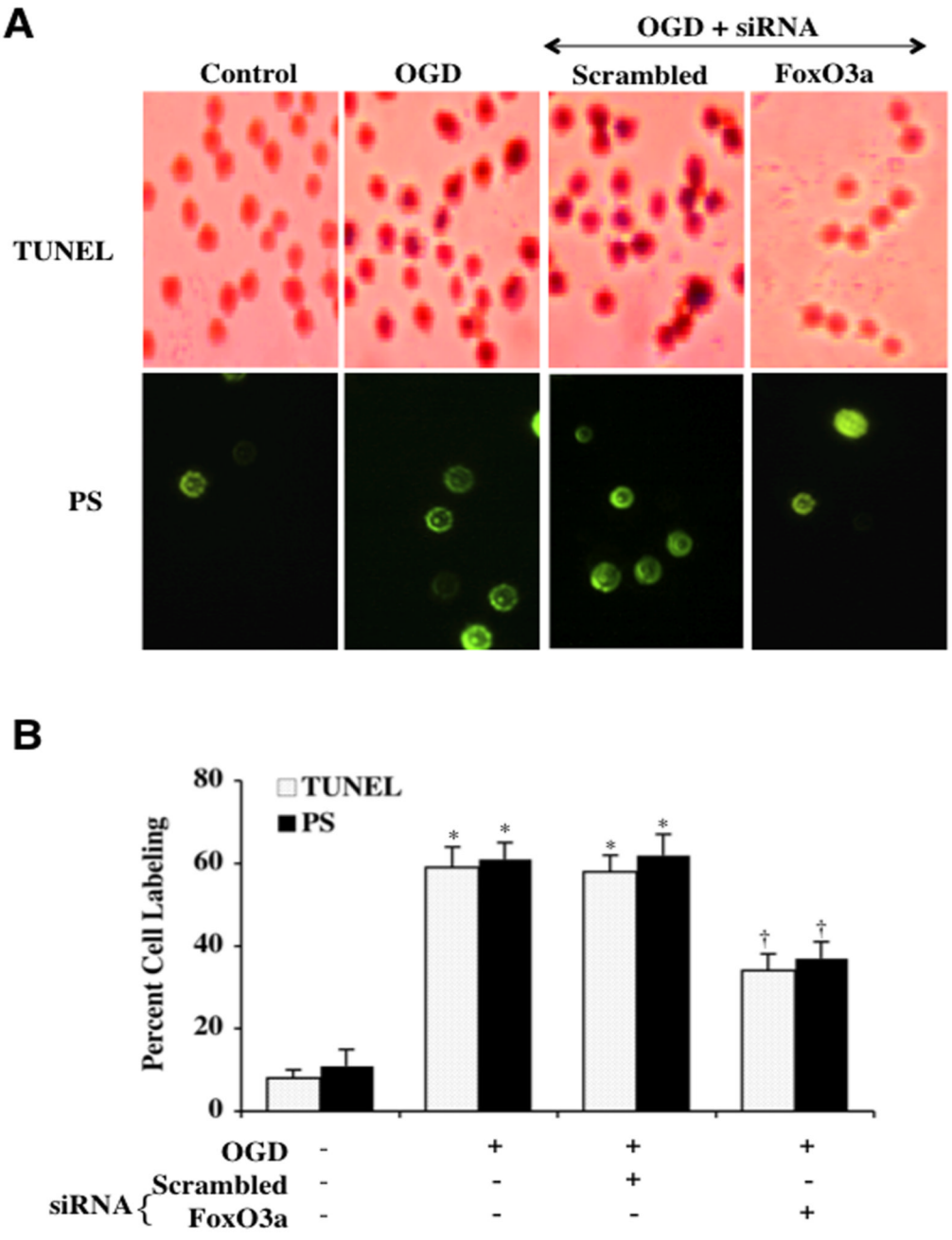

Fig. (5). Transient gene knockdown of *FoxO3a* limits apoptotic DNA fragmentation and phosphatidylserine (PS) exposure

In **A** and **B**, representative images and quantitative analysis illustrate transient gene knockdown of *FoxO3a* with FoxO3a siRNA (siRNA) significantly blocked microglial genomic DNA degradation assessed by TUNEL and membrane PS externalization assessed by annexin V (green fluorescence) 24 hours after OGD (**P*<0.01 *vs*. untreated cells; †*P* <0.01 *vs*. OGD). FoxO3a siRNA alone was not toxic and non-specific scrambled siRNA did not protect cells during OGD (**P*<0.01 *vs*. untreated cells). Each data point represents the mean and SEM from 6 experiments.

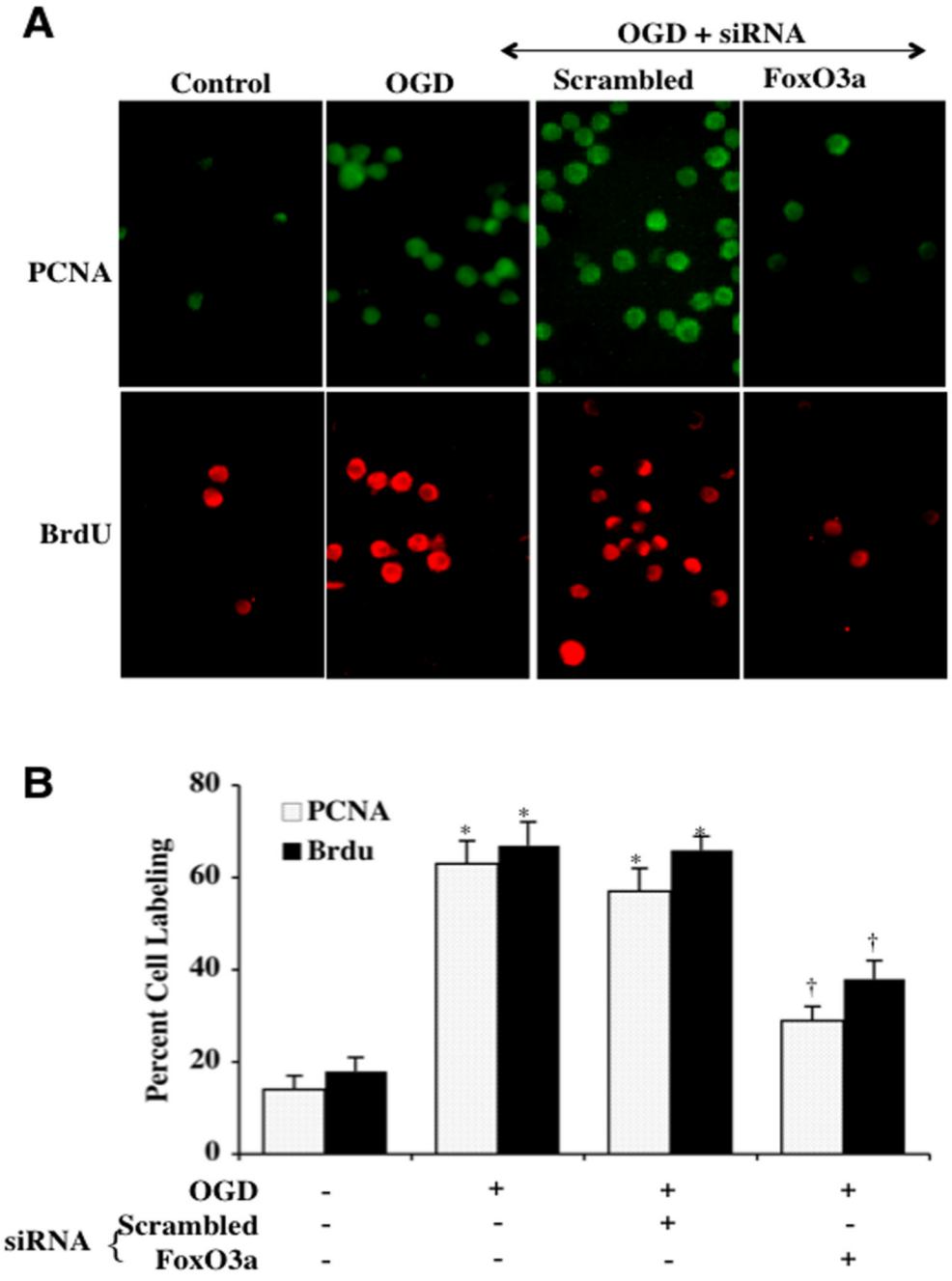

Fig. (6). Absence of FoxO3a protein prevents the early activation and proliferation of microglia
In **A** and **B**, representative images and quantitative analysis demonstrate that transient gene knockdown of *FoxO3a* with FoxO3a siRNA (siRNA) decreases the expression of PCNA and the uptake of BrdU at 6 hours after OGD (**P*<0.01 *vs*. untreated cells; †*P* <0.01 *vs*. OGD). FoxO3a siRNA alone was not toxic and non-specific scrambled siRNA did not alter PCNA expression or BrdU uptake during OGD (**P*<0.01 vs. untreated cells). In all cases, control = untreated cells. Each data point represents the mean and SEM from 6 experiments.

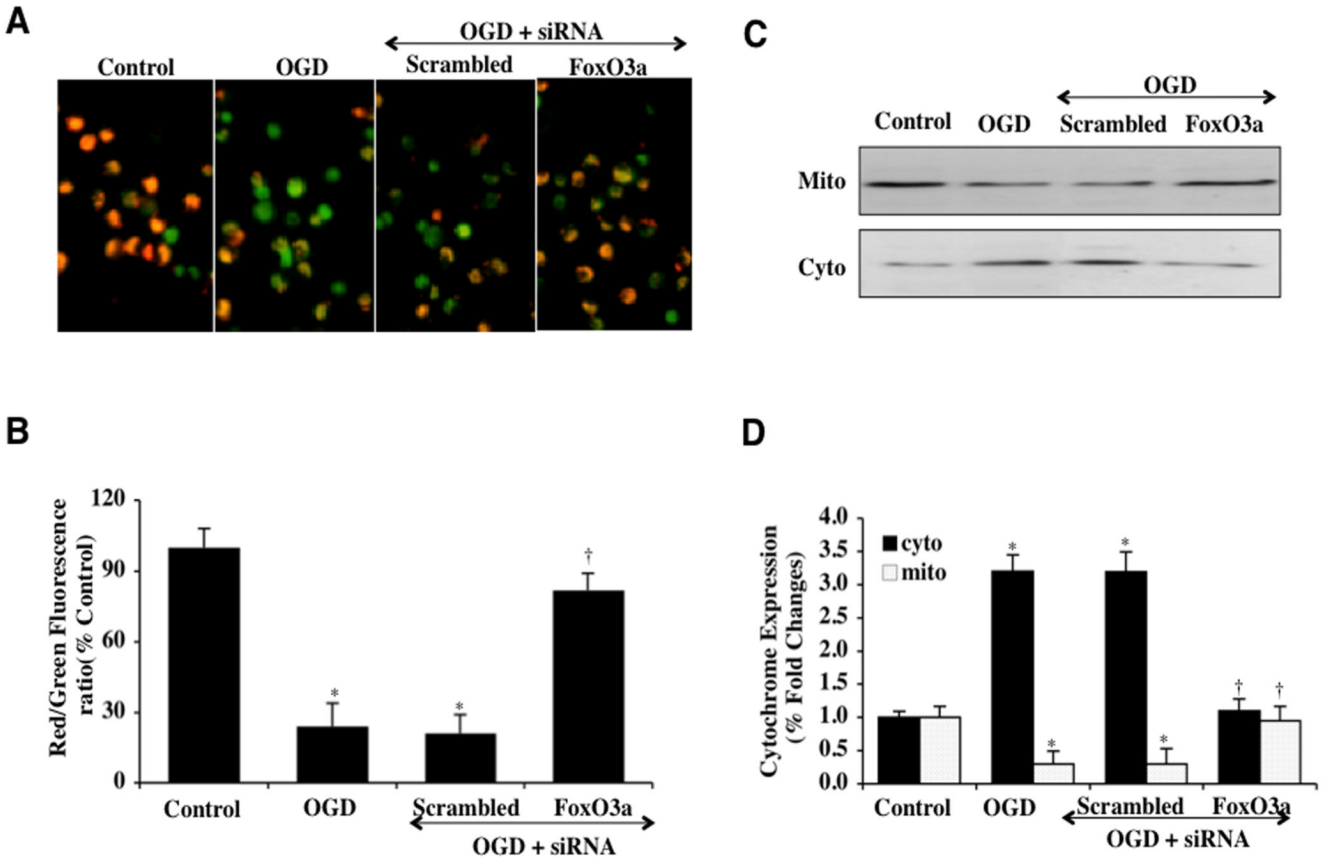

Fig. (7). Transient gene knockdown of *FoxO3a* prevents mitochondrial depolarization and the release of mitochondrial cytochrome c

(**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. Transient gene knockdown of *FoxO3a* with FoxO3a siRNA (siRNA) during OGD significantly increased the red/green fluorescence intensity of mitochondria in microglia, indicating that membrane potential was restored. Non-specific scrambled siRNA did not prevent mitochondrial membrane depolarization. (**B**) The relative ratio of red/green fluorescent intensity of mitochondrial staining in both untreated (control) microglia and microglia exposed to OGD or transfected with FoxO3a siRNA was measured in 6 independent experiments with analysis performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image) (Control *vs*. OGD, **P*<0.01; OGD *vs*. FoxO3a siRNA, †*P*<0.01). (**C** and **D**) A representative Western blot with equal amounts of mitochondrial (mito) or cytosol (cyto) protein extracts (50 μg/lane) were immunoblotted demonstrating that transfection of FoxO3a siRNA significantly prevented cytochrome c release from mitochondria during OGD (**P*<0.01 *vs*. Control; †*P* <0.01 *vs*. OGD). Non-specific scrambled siRNA did not prevent mitochondrial depolarization. Each data point represents the mean and SEM from 6 experiments.

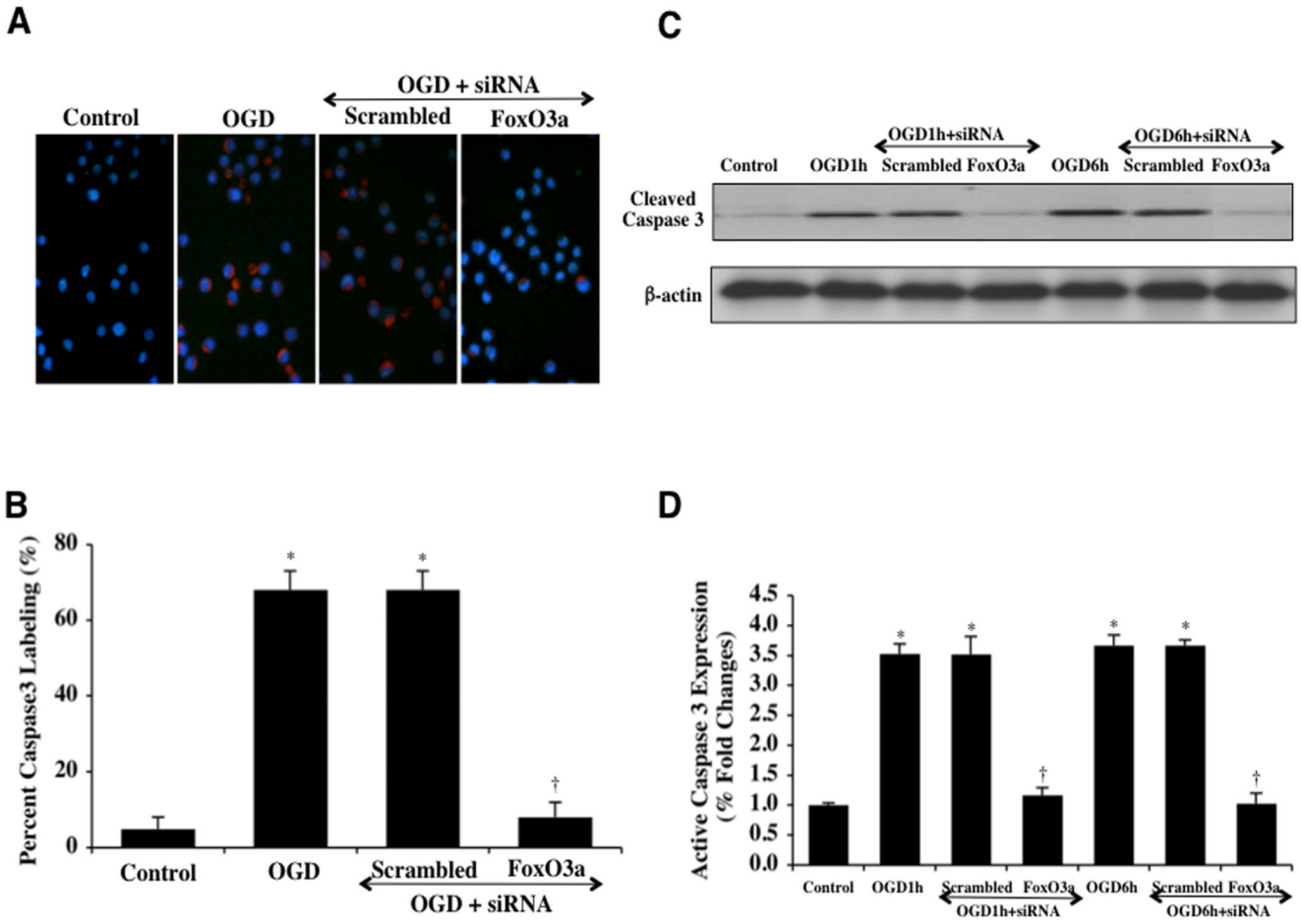

Fig. (8). FoxO3a modulates caspase 3 activity during OGD

In **A** and **B**, microglial cells were exposed to OGD and caspase 3 activation was determined 6 hours after OGD exposure through immunocytochemistry with antibodies against cleaved active caspase 3 (17 kDa). Representative images illustrate active caspase 3 staining (red) in cells following OGD in which red staining is almost absent during transfection with FoxO3a siRNA. Non-specific scrambled siRNA does not eliminate caspase 3 activity. Quantification of data demonstrates that OGD significantly increased the expression of cleaved active caspase 3 when compared to untreated control cells (**P*<0.01 vs. control). Yet, the expression of cleaved active caspase 3 was significantly decreased in cells with transfection of FoxO3a siRNA for 3 days prior to the exposure to OGD (**P* <0.01 *vs*. Control; †*P*<0.01 *vs*. OGD). In **C** and **D**, microglial protein extracts (50 μg/lane) were immunoblotted with anti-cleaved caspase 3 product (active caspase 3, 17 kDA) at 1 hour (1h) and 6 hours (6h) following OGD. OGD significantly increased cleaved caspase 3 expression, but transient gene knockdown of *FoxO3a* with FoxO3a siRNA (siRNA) significantly prevented cleaved caspase 3 expression 1 hour and 6 hours after OGD. FoxO3a siRNA alone was not toxic and non-specific scrambled siRNA did not reduce cleaved caspase 3 expression during OGD (**P* <0.01 *vs*. Control; †*P*<0.01 vs. OGD). In all cases, each data point represents the mean and SEM from 6 experiments.

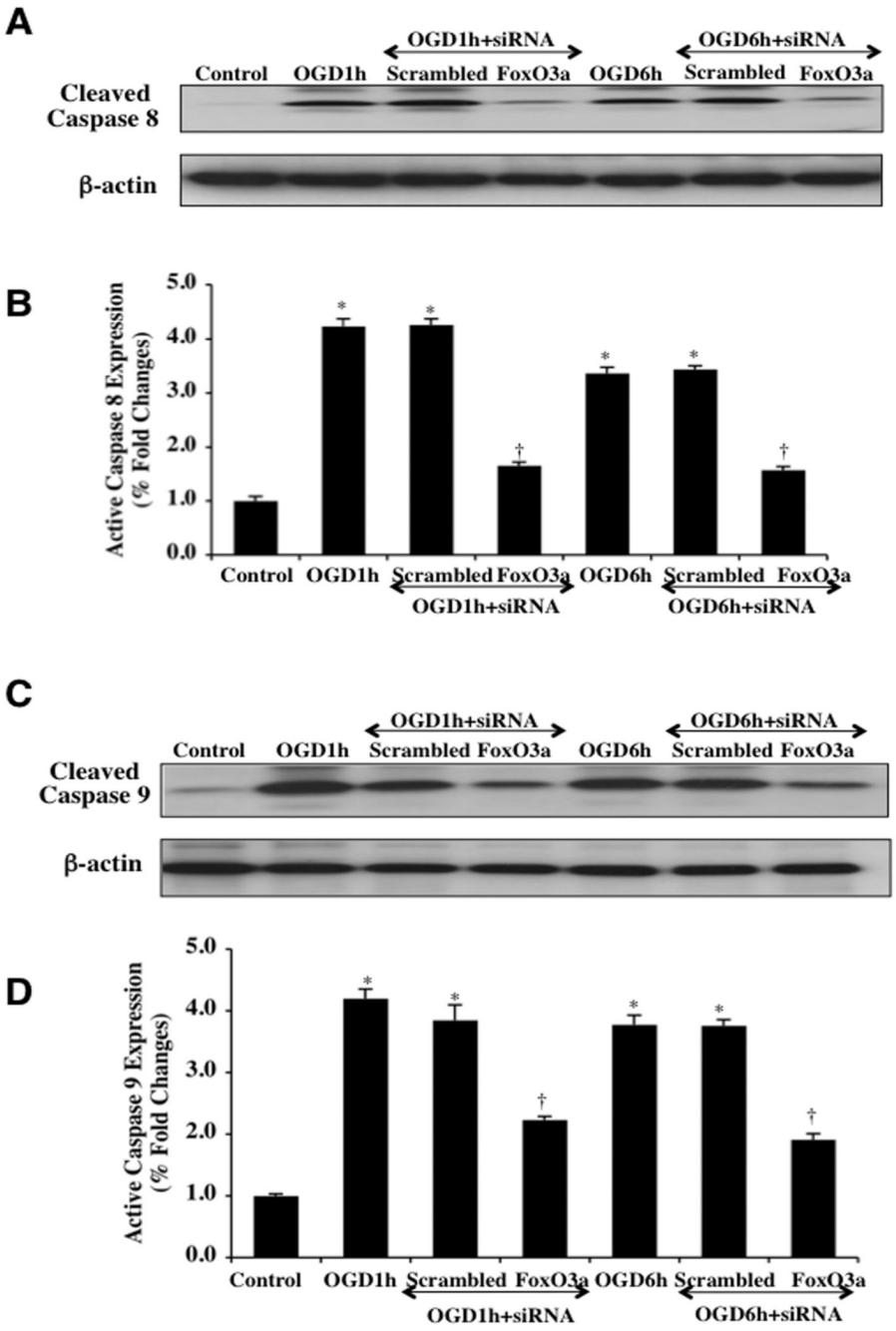

Fig. (9). Caspase 8 and 9 activities are controlled by FoxO3a during OGD

In **A** and **B**, microglial protein extracts (50 μg/lane) were immunoblotted with anti-cleaved caspase 8 product (active caspase 8, 18 kDA) at 1 hour (1h) and 6 hours (6h) following OGD. OGD markedly increased cleaved caspase 8 expression, but transient gene knockdown of *FoxO3a* with FoxO3a siRNA (siRNA) significantly blocked cleaved caspase 8 expression 1 hour and 6 hours after OGD. FoxO3a siRNA alone was not toxic and non-specific scrambled siRNA did not reduce cleaved caspase 8 expression during OGD (**P* <0.01 *vs*. Control; †*P*<0.01 *vs*. OGD). In all cases, each data point represents the mean and SEM from 6 experiments. In **C** and **D**, microglial protein extracts (50 μg/lane) were immunoblotted with anti-cleaved caspase 9 product (active caspase 9, 37 kDA) at 1 hour (1h) and 6 hours (6h)

following OGD. OGD markedly increased cleaved caspase 9 expression, but transient gene knockdown of *FoxO3a* with FoxO3a siRNA (siRNA) limited cleaved caspase 9 expression 1 hour and 6 hours after OGD. FoxO3a siRNA alone was not toxic and non-specific scrambled siRNA did not reduce cleaved caspase 9 expression during OGD (**P* <0.01 *vs*. Control; †*P*<0.01 *vs*. OGD). In all cases, each data point represents the mean and SEM from 6 experiments.