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WISP1 (CCN4) Autoregulates its Expression and Nuclear Trafficking of β-Catenin during Oxidant Stress with Limited Effects upon Neuronal Autophagy

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Abstract

Wnt1 inducible signaling pathway protein 1 (WISP1/CCN4) is a CCN family member more broadly identified with development and tumorigenesis. However, recent studies have shed new light and enthusiasm on WISP1 as a novel target directed against toxic cell degeneration. Here we show WISP1 prevents apoptotic degeneration in primary neurons during oxidant stress through the activation of protein kinase B (Akt1), the post-translational maintenance of β-catenin integrity that is consistent with inhibition of glycogen synthase kinase-3β (GSK-3β), and the subcellular trafficking of β-catenin to foster its translocation to the nucleus. Interestingly, WISP1 autoregulates its expression through the promotion of β-catenin activity and may employ β-catenin to have a limited control over autophagy, but neuronal injury during oxidant stress as a result of autophagy appears portioned to a small population of neurons without significant impact upon overall cell survival. New strategies that target WISP1, its autoregulation, and the pathways responsible for neuronal cell injury may bring forth new insight for the treatment of neurodegenerative disorders.

Keywords

Akt1; apoptosis; autophagy; β-catenin; Beclin 1; CCN4; glycogen synthase kinase-3β; LC3; neurons; oxidative stress; p62; WISP; Wnt1

INTRODUCTION

Wnt1 inducible signaling pathway protein 1 (WISP1) was initially identified as a gene in a mouse mammary epithelial cell line [1], later determined to modulate gastric tumor growth [2], and is regulated by Wnt1 [1], a cysteine-rich glycosylated protein that controls cell growth, development, and survival in multiple systems of the body [3–11]. WISP1, also known as CCN4, is part of the CCN family of proteins that is defined by the first three members of the family that include Cysteine-rich protein 61, Connective tissue growth factor, and Nephroblastoma overexpressed gene [12]. The CCN family is characterized by

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CONFLICTS OF INTEREST

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four cysteine-rich modular domains that include insulin-like growth factor-binding domain, von Willebrand factor type C module, thrombospondin domain, and C-terminal cysteine knot-like domain [13].

WISP1 has been associated with neoplastic growth [14, 15] and early studies have demonstrated that WISP1 can block p53 mediated DNA damage and apoptosis [16]. However, subsequent work has shown that WISP1 may be beneficial as a proliferative agent. WISP1 may be involved with cardiac remodeling following myocardial infarction [17], with lung tissue repair and re-growth [18], with the stimulation of vascular smooth muscle growth [19], and with the induction of cardiomyocyte proliferation [20]. In addition, WISP1 is cytoprotective against cardiac toxicity [21], may foster bone formation and fracture repair [22, 23], and can limit neuronal cell injury during oxidative stress [24].

WISP1 utilizes several signal transduction pathways to yield cellular proliferation, survival, and repair that include the modulation of downstream apoptotic signaling involving mitochondrial stress, cytochrome c release, and caspase activation [16, 21, 24, 25]. Further upstream from these pathways, WISP1 can prevent cell injury through the activation of phosphoinositide 3-kinase (PI 3-K) and Akt1, primary mediators of cellular growth and survival [3, 26–31]. WISP1 phosphorylates Akt under conditions of cardiac tissue injury, oxidative stress, neuronal degeneration, and vascular smooth muscle proliferation [16, 19, 21, 24]. With the phosphorylation of Akt, WISP1 also leads to the inhibitory phosphorylation of glycogen synthase kinase-3β (GSK-3β) [21, 24] and the nuclear expression of β-catenin in cardiomyocytes [21]. Interestingly, β-catenin through Wnt signaling promotes the expression of WISP1 [32]. During the inhibition of GSK-3β, β-catenin is not phosphorylated, ubiquinated, or degraded and therefore can translocate to the nucleus to initiate “antiapoptotic” pathways and prevent cellular apoptosis [3, 33–39]. Furthermore, β-catenin also may function to limit cell injury through the blockade of autophagy [40, 41].

WISP1 and its signaling pathways with β-catenin represent a novel target that has the potential to promote tissue proliferation, repair, and regeneration in multiple cell systems, especially the central nervous system. We show in primary hippocampal neurons that oxygen-glucose deprivation (OGD) primarily leads to apoptotic cell death and that autophagy represents a smaller subset of the neuronal cell death observed. In primary neurons, WISP1 prevents apoptotic neuronal cell death, phosphorylates Akt1, limits β-catenin phosphorylation through the suggested inhibition of GSK-3β, and fosters the subcellular trafficking of β-catenin from the cytoplasm to the cell nucleus. β-catenin is necessary for WISP1 to prevent neuronal apoptotic degeneration and to autoregulate its expression, but β-catenin may play a lesser role in OGD mediated neuronal autophagy since WISP1 through β-catenin has limited effects upon the lipidation of light chain 3 -I (LC3-I) to LC3-II, the expression of Beclin 1, and the expression of p62 during the induction of autophagy with OGD.

MATERIALS AND METHODS

Hippocampal Neuronal Cultures

Per our prior protocols [4, 24, 42–44], hippocampi were obtained from E-19 Sprague-Dawley rat pups and incubated in Hanks’ balanced salt solution (HBBS) supplemented with 1 mM sodium pyruvate and 10 mM HEPES buffer solution (Invitrogen, Carlsbad, CA). The neurons were isolated by trituration for 10 times, centrifuged for 2 min at 200 g and then dissociated in growth medium (Leibovitz's L-15 medium, Invitrogen, Carlsbad, CA) containing 6% sterile rat serum (ICN, Aurora, OH), 150 mM $NaHCO_3$, 2.25 mg/ml of transferrin, 2.5 μg/ml of insulin, 10 nM progesterone, 90 μM putrescine, 15 nM selenium, 35

mM glucose, 1 mM L-glutamine, penicillin and streptomycin (50 μg/ml), and vitamins. Cells were then plated at a density of ~1.5 $\times 10^3$cells/mm^2 in 35 mm polylysine/laminin-coated plates (Falcon Labware, Lincoln Park, NJ). Neurons were maintained in growth medium at 37 °C in a humidified atmosphere of 5% CO_2 and 95% room air for 10–14 days.

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 our prior experimental protocols [29, 45, 46). For treatments applied prior to OGD, human recombinant WISP1 protein (R&D Systems, Minneapolis, MN) was continuous. The phosphatidylinositol-3-kinase (PI3-K) inhibitors wortmannin (0.5 μM, Calbiochem, La Jolla, CA) and LY294002 (10 μM, Sigma, St Louis, MO), the autophagy inhibitor 3-methyladenine) 3MA))10 mM, Sigma, St Louis, MO), the β-catenin agonist 2-amino-4-)3,4-)methylenedioxy) benzyl-amino)-6-)3-methoxyphenyl)pyrimidine (AMBP, 1–20 μM, EMD, Billerica, MA), and the β-catenin antagonist 2-phenoxybenzoic acid-(5-methyl-2-furanyl) methylene (hydrazide) PNU74654, PNU, 1–10 μM, R&D Systems, Minneapolis, MN) were each administered directly to the cultures 1 hour prior to OGD and treatments were continuous.

Assessment of Cell Survival

Neuronal cell injury was determined by bright field microscopy using a 0.4% trypan blue dye exclusion method 24 hours following treatment with OGD per our previous protocols [27, 46, 47]. 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 4–6 times with different cultures

Assessment of DNA Fragmentation

Genomic DNA fragmentation was determined by the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay [48–50]. Briefly, neuronal 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).

Expression of Phosphorylated Akt1, Beclin 1, p62, β-Catenin, WISP1, and LC3

Cell protein extracts were subjected to SDS-PAGE (7.5%, Akt1, Beclin 1, p62, and β-catenin; 12.5%, WISP1 and LC3) separation. After blocking for 1 hour at room temperature with 5% skim milk, the membranes were incubated overnight at 4 °C with a rabbit polyclonal antibody against WISP1 (1:200, Santa Cruz Biotechnologies, Santa Cruz, CA), a rabbit polyclonal antibody against phospho-Akt1 (p-Akt1, Ser^{473}, 1:1000, Cell Signaling, Beverly, MA), a rabbit monoclonal antibody against phospho-Beclin 1 (1:1000), a rabbit polyclonal antibody against phospho-β-catenin (p-β-catenin, Ser^{33,37}, 1:1000), a rabbit polyclonal antibody against p62 (1:1000), and a rabbit polyclonal antibody against LC3 (1:1000) (Cell Signaling, Beverly, MA). After incubation of the membranes with horseradish peroxidase conjugated secondary antibody (goat anti-mouse IgG, 1:5000) (Pierce, Rockford, IL), the antibody-reactive bands were revealed by enhanced chemiluminescence detection on Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ).

Western blot analysis for β-Catenin in the cytoplasm and the nucleus: Cells were homogenized, the cytoplasmic and nuclear proteins were prepared by using NE-PER nuclear and cytoplasmic extraction reagents according to manufacture's instructions (Pierce, Rockford, IL). The expression of β-catenin in nucleus and cytoplasm was determined by Western blot. Each sample (50 μg/lane) was subjected to 7.5% SDS-polyacrylamide gel electrophoresis and Western blot was performed as description as above.

Immunocytochemistry for β-Catenin

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

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

WISP1 Reduces Neuronal Apoptosis during Oxygen Glucose Deprivation (OGD)

Recombinant human WISP1 protein (10 ng/ml) was administered to neuronal cultures 1 hour prior to a 3 hour period of OGD and cell injury was determined 24 hours later through trypan blue dye exclusion method and TUNEL assay. This concentration of WISP1 was chosen since it previously was shown to provide significant cytoprotection in neuronal cells and to significantly lead to the phosphorylation of Akt1 (Ser^{473}) [24]. In Fig. (**1A**), representative images demonstrate that untreated neurons were not significantly stained with trypan blue and TUNEL, but exposure to OGD resulted in significant trypan blue staining and nuclear DNA damage 24 hours following OGD exposure in neurons. In contrast, WISP1 (10 ng/ml) significantly reduced trypan blue staining and nuclear DNA degradation. Quantitative results illustrate indicate that percent trypan blue staining and DNA fragmentation were significantly increased in neurons 24 hours following OGD (Fig. **1B**). In contrast, WISP1 (10 ng/ml) treatment significantly reduced percent trypan blue staining and DNA fragmentation.

WISP1 Regulates Phosphorylation of β-Catenin through the PI 3-K/Akt1 Pathway during OGD

To assess the ability of WISP1 to phosphorylate Akt1, western blot assay was performed for phosphorylated Akt1 (p-Akt1) (Ser^{473}) (activated form of Akt1) at 3 hours after administration of WISP1 (10 ng/ml) to neurons. As shown in Fig. 1C, WISP1 significantly increased p-Akt1 expression at 3 hours following OGD exposure. However, this increase in the expression of p-Akt1 was abrogated by the PI 3-K inhibitors wortmannin (0.5 μM) and LY294002 (10 μM) during treatment with WISP1 (10 ng/ml) (Fig. **1C**). Wortmannin (0.5 μM) forms a covalent link with the lysine residue of PI 3-K [51] and LY294002 (10 μM) reversibly competes for ATP binding [52].

Since β-catenin is the downstream target of Wnt/GSK-3β and Akt signaling pathways [53, 54], we next examined the effects of WISP1 on β-catenin phosphorylation that can lead to β-catenin ubiquination and degradation. In Fig. (**1D**), expression of p-β-catenin ($Ser^{33/37}$) was significantly increased 3 hours following OGD exposure. In contrast, WISP1 (10 ng/ml) applied 1 hour prior to a 3 hour period of OGD significantly prevented the expression of p-β-catenin 3 hours following OGD exposure (Fig. **1D**). The ability of WISP1 to block phosphorylation of β-catenin was prevented during treatment with the PI 3-K inhibitors wortmannin (0.5 μM) and LY294002 (10 μM), illustrating that the PI 3-K pathway is utilized by WISP1 to control β-catenin phosphorylation. In addition, the phosphorylation of β-catenin was prevented during the administration of WISP1 (10 ng/ml) with the GSK-3β inhibitor SB21673 (5 μM) to a similar degree as observed with application of WISP1 alone during OGD, suggesting that blockade of β-catenin phosphorylation during OGD exposure was also mediated by GSK-3β inhibition.

WISP1 through the PI 3-K Pathway Promotes the Nuclear Translocation of β-Catenin in Neurons during OGD

β-catenin that is not phosphorylated can translocate to the nucleus and foster protection against apoptosis [3, 35, 45, 55–58] and autophagy [40, 41, 59]. We therefore investigated the ability of WISP1 to regulate the translocation of β-catenin in neurons during OGD exposure. WISP1 (10 ng/ml) was applied to neuronal cultures 1 hour prior to a 3 hour period of OGD and western blot analysis for β-catenin in the extracts from both nucleus and cytoplasm was performed. As shown in Fig. (**2A**), OGD resulted in an increase in the expression of β-catenin in the cytoplasm and a decrease in the expression of β-catenin in the nucleus. In contrast, WISP1 treatment significantly increased the expression of β-catenin in the nuclei of neurons. Application of the PI 3-K inhibitor LY294002 (10 μM) prevented the nuclear translocation of β-catenin during WISP1 administration, suggesting that the PI 3-K pathway was necessary for WISP1 to traffic β-catenin. Immunofluorescence staining for β-catenin (Texas-red) was performed at 3 hours following OGD and nuclei of neurons were counterstained with DAPI (Fig. **2B**). In merged images, cells with OGD alone demonstrate neuronal nuclei with minimal β-catenin staining (blue/white) and neuronal cytoplasms with significant β-catenin staining (red). This is in contrast to neurons treated with WISP1 that demonstrate significant increase in nuclear staining of β-catenin. Similar to the western analysis studies, administration of the PI 3-K inhibitor LY294002 (10 μM) prevented the nuclear translocation of β-catenin during WISP1 exposure. The immunofluorescence studies with β-catenin parallel the western blot studies for β-catenin expression in the cytoplasm and the nucleus (Fig. **2A**).

WISP1 Protects Neurons through β-Catenin Activation during OGD Exposure

The β-catenin agonist AMBP (1, 10, and 20 μM) was applied to neuronal cultures 1 hour prior to a 3 hour period of OGD and cell injury was determined 24 hours following OGD exposure through trypan blue (TB) dye exclusion and apoptotic nuclear DNA fragmentation (TUNEL). AMBP (20 μM) significantly reduced trypan blue uptake and DNA fragmentation in neuronal cells (Fig. **3A**) and the β-catenin inhibitor PNU (20 μM) significantly increased neuronal cell injury (Fig. **3B**). Quantitative results demonstrate that the percent cell labeling of typan blue and TUNEL was significantly decreased by the treatment with the β-catenin agonist. In contrast, application of β-catenin inhibitor PNU (20 μM) increased trypan blue staining and DNA fragmentation during OGD exposure when compared with neurons treated with OGD alone (Figs. **3A** and **3B**). In addition, application of PNU (20 μM) with WISP1 significantly reduced the ability of WISP1 to protect cells during OGD exposure, suggesting that WISP1 employs β-catenin to foster neuronal protection (Fig. **3B**).

WISP1 Autoregulates its Expression through β-Catenin During OGD

Increased activity of β-catenin can promote WISP1 expression in cells exclusive of the nervous system [9, 21, 60]. Given that WISP1 can prevent the phosphorylation of β-catenin and translocate this protein to the cell nucleus in primary neurons, we examined whether β-catenin could then regulate the expression of WISP1 as a feedback mechanism. Hippocampal neuronal protein extracts (50 μg/lane) were immunoblotted with anti-WISP1 antibody at 24 hours following a 3 hour period of OGD. As shown in Fig. (**4A**), WISP1 can lead to the induction of its own expression. WISP1 expression was increased in control untreated neurons and in neurons exposed to OGD 24 hours following OGD exposure. Application of WISP1 (10 μM) 1 hour prior to OGD significantly increased the expression of WISP1 in neurons 24 hours following OGD exposure.

We next assessed whether WISP1 can regulate its expression through β-catenin. Wnt1 protein (100 ng/ml), the β-catenin agonist AMBP (20 μM), or the β-catenin inhibitor PNU (20 μM) were applied to neuronal cultures 1 hour prior to a 3 hour period of OGD (Figs. **4B** and **4C**). The cysteine-rich glycosylated protein Wnt1, known to lead to β-catenin activation [54, 61], was also used in these studies, since it has previously been shown that Wnt1 can increase WISP1 expression in primary neurons [24]. Western blot analysis was performed for WISP1 24 hours following OGD. Both Wnt1 and the β-catenin agonist AMBP significantly increased WISP1 expression during OGD exposure. In contrast, WISP1 expression was significantly reduced during application of the β-catenin inhibitor PNU (20 μM) alone or during WISP1 administration, illustrating that β-catenin activity is necessary for WISP1 expression.

Autophagy has a Limited Role for Neuronal Survival during OGD Exposure

To investigate the regulatory effects of autophagy on neuronal survival during OGD, we applied the autophagy inhibitor 3MA (10 mM) to neuronal cultures 1 hour prior to a 3 hour period of OGD. Neuronal cell injury was determined 24 hours following OGD by using the trypan blue dye exclusion method. In Fig. (**5A**), representative images demonstrate that untreated neurons were without evidence of trypan blue uptake, but exposure to OGD resulted in significant trypan blue staining 24 hours following OGD in neurons. Application of the autophagy inhibitor 3MA did not significantly affect neuronal cell injury during OGD exposure. Quantitative results show that percent trypan blue staining (Fig. **5A**) were not significantly affected by 3MA treatment in neurons 24 hours following OGD, suggesting that autophagy has a minimal role for cell survival in possibly only a subset of primary neurons during OGD exposure.

WISP1 through β-Catenin has a Minimal Effect upon Autophagy Parameters during OGD Exposure

For our analysis of autophagic flux, we assessed LC3-II, Beclin-1, and p62 expression, since the presence of LC3-II alone may not always correlate with autophagy activity [62]. Hippocampal neuronal protein extracts were immunoblotted with anti- microtubule-associated protein 1 light chain 3 (LC3), p62, and Beclin-1 antibodies at 1, 3, and 24 hours following a 3 hour period of OGD. OGD exposure resulted in increased expression of LC3-II (autophagosome-specific) and Beclin-1, but an expected decrease in p62 expression, consistent with the induction of autophagy in primary neurons (Fig. **5B**). Administration of WISP1 (10 ng/ml) to neuronal cultures 1 hour prior to OGD resulted in a minor increase in p62 expression without significant changes in LC3-II and Beclin-1 expressions. Application of 3MA as a control during OGD significantly prevented increased expression of LC3-II and Beclin-1 and blocked reduction in p62 expression, consistent with the prevention of autophagy (Fig. **5C**). The β-catenin agonist AMBP (20μM) was applied to neuronal cultures 1 hour prior to OGD and the expressions of LC3-II, Beclin-1, and p62 were determined 24

hours following a 3 hour period of OGD. Activation of β-catenin with AMBP decreased the expressions of LC3-II and Beclin-1 while increasing p62 expression to a greater degree that WISP1 alone 24 hours following OGD exposure, suggesting that the limited response of WISP1 against autophagy may be mediated in part through β-catenin activation.

DISCUSSION

WISP1 has a broad role in multiple systems of the body that include bone formation and repair [22, 23], tumorigenesis and aggressive cancer progression in the gastric system [2, 15], and cellular hypertrophy [20]. WISP1 also can enhance cell survival during cardiomyocyte injury [21, 63], potentially limit vascular cell death during crush injury in human saphenous veins [64], and block primary neuronal early and late apoptotic injury during oxidative stress [24]. We show that exogenous application of WISP1 prevents neuronal cell injury and blocks apoptotic DNA degradation during OGD exposure consistent with prior studies illustrating that WISP1 can prevent cardiomyocyte death against tumor necrosis factor [63], increase survival in human lung carcinoma cells during UV irradiation and etoposide treatment [16], and block neuronal degeneration during oxidant stress [24].

WISP1 provides neuronal cellular protection against oxidative stress during OGD exposure through the phosphorylation and activation of Akt1 and the prevention of β-catenin phosphorylation that can occur through GSK-3β and lead to the degradation of β-catenin. During oxidative stress, Akt1 activation can protect and promote the proliferation of endothelial cells [3, 27–29, 31, 47, 65–70], lead to enhanced survival and activity of glial cells [10, 11, 30, 35, 45, 71–73], foster cardiac tissue protection [74, 75], and prevent the degeneration of neurons [4, 24, 76–82]. We show that WISP1 phosphorylates Akt1 and subsequently prevents the phosphorylation of β-catenin through a PI 3-K mediated pathway. In addition, GSK-3β inhibition with WISP1 application did not synergistically decrease the phosphorylation of β-catenin, suggesting that prevention of β-catenin phosphorylation by WISP1 during OGD exposure was also mediated by GSK-3β inhibition. Inhibition of GSK-3β is known to prevent β-catenin phosphorylation [3, 33–36, 83–85].

WISP1 also controls the subcellular trafficking of β-catenin. In osteoclasts [60], vascular cells [9], and cardiomyocytes [21], WISP1 has been associated with increased nuclear expression of β-catenin. We show that WISP1 through a PI 3-K mediated pathway in primary neurons fosters the translocation of β-catenin from the cytoplasm of neurons to the nucleus that can allow for the transcription and eventual translation of pathways that can limit apoptosis [3, 35, 45, 55–58] and autophagy [40, 41, 59]. In addition, WISP1 requires β-catenin to limit neuronal cell injury during OGD exposure, since inhibition of β-catenin activity during OGD increases cell injury and blocks neuroprotection against apoptosis by WISP1.

The ability of WISP1 to control the phosphorylation and cellular trafficking of β-catenin appears to be necessary for WISP1 to autoregulate its expression. Increased activity of β-catenin can promote WISP1 expression in osteoclasts and vascular cells [9, 60] and administration of exogenous WISP1 can increase the expression of endogenous WISP1 in cardiomyocytes [21]. In primary hippocampal neurons, we demonstrate that exogenous application of WISP1 not only increases WISP1 in untreated control neurons, but also significantly increases WISP1 expression during oxidative stress exposure. As a result, WISP1 can lead to the induction of its own expression. Furthermore, we show that loss of β-catenin activity leads to depressed WISP1 expression while increased β-catenin activity promotes WISP1 expression, suggesting that WISP1 expression is governed by β-catenin activity and that WISP1 regulates its own expression through the ability of WISP1 to control β-catenin phosphorylation and nuclear translocation.

However, the ability of WISP1 to control β-catenin activity does not appear to alter autophagy progression in primary neurons during OGD exposure. Our studies demonstrate that inhibition of autophagy with 3MA does not significantly protect neurons during OGD, suggesting that autophagy may play a small role in acute neuronal injury during oxidative stress. Furthermore, during OGD exposure, soluble cytosolic LC3-I is lipidated to the autophagosome bound LC3-II per western analysis that indicates activation of the autophagy pathways. Since the presence of LC3-II may not always correlate with autophagy activity [62], we also assessed Beclin-1 expression [86, 87] and p62 expression [88] to evaluate autophagic flux. We show that Beclin-1 expression is increased during OGD exposure over time, but that p62 expression is decreased, suggesting that autophagy occurs in at least a small subset of neurons during OGD exposure. Yet, WISP1 does not significantly alter LC3-II, Beclin-1, or p62 expression when compared to neurons exposed only to OGD, illustrating that WISP1 with the concentration that we employed does not significantly modulate autophagy during OGD exposure in primary neurons. Administration of the β-catenin agonist AMPB did more prominently alter autophagy in neurons, suggesting that in this model of neuronal oxidative stress β-catenin appears to have some influence to limit autophagy. When examining the three parameters used to assess autophagy, WISP1 was able to increase p62 expression without significant alterations to LC3-II and Beclin-1. β-catenin activation had a more effective response in decreasing the expressions of LC3-II and Beclin-1 and increasing p62 expression when compared to neurons exposed to OGD alone, suggesting that WISP1 may, in part, slightly limit autophagy through β-catenin activation.

As a member of the Wnt signaling pathway, WISP1 may play an important role in both development and neoplastic growth [8, 12]. However, the capacity of WISP1 to promote cell survival during toxic environments is generating a new line of excitement for this cellular target. Although WISP1 may rely upon PI 3-K, mitochondrial, and cytokine pathways to elicit increased cell survival [16, 21, 24, 63], WISP1 also has been associated with β-catenin activity [9, 60]. Our studies highlight the vital roles Akt1 and β-catenin hold for WISP1 not only to autoregulate the expression of WISP1, but also for WISP1 to prevent neuronal cell injury during oxidant stress. Protection by WISP1 in neuronal cells is primarily directed against apoptotic cell death with minimal influence against autophagic cell injury, suggesting that the Akt1 pathway may be central for WISP1 to control multiple cell survival pathways that include mitochondria and β-catenin. Novel therapies that can focus upon WISP1 (CCN4), its autoregulation, and the cytoprotective pathways controlled by this CCN family member may offer unique advantages for the treatment of degenerative disorders in the nervous system.

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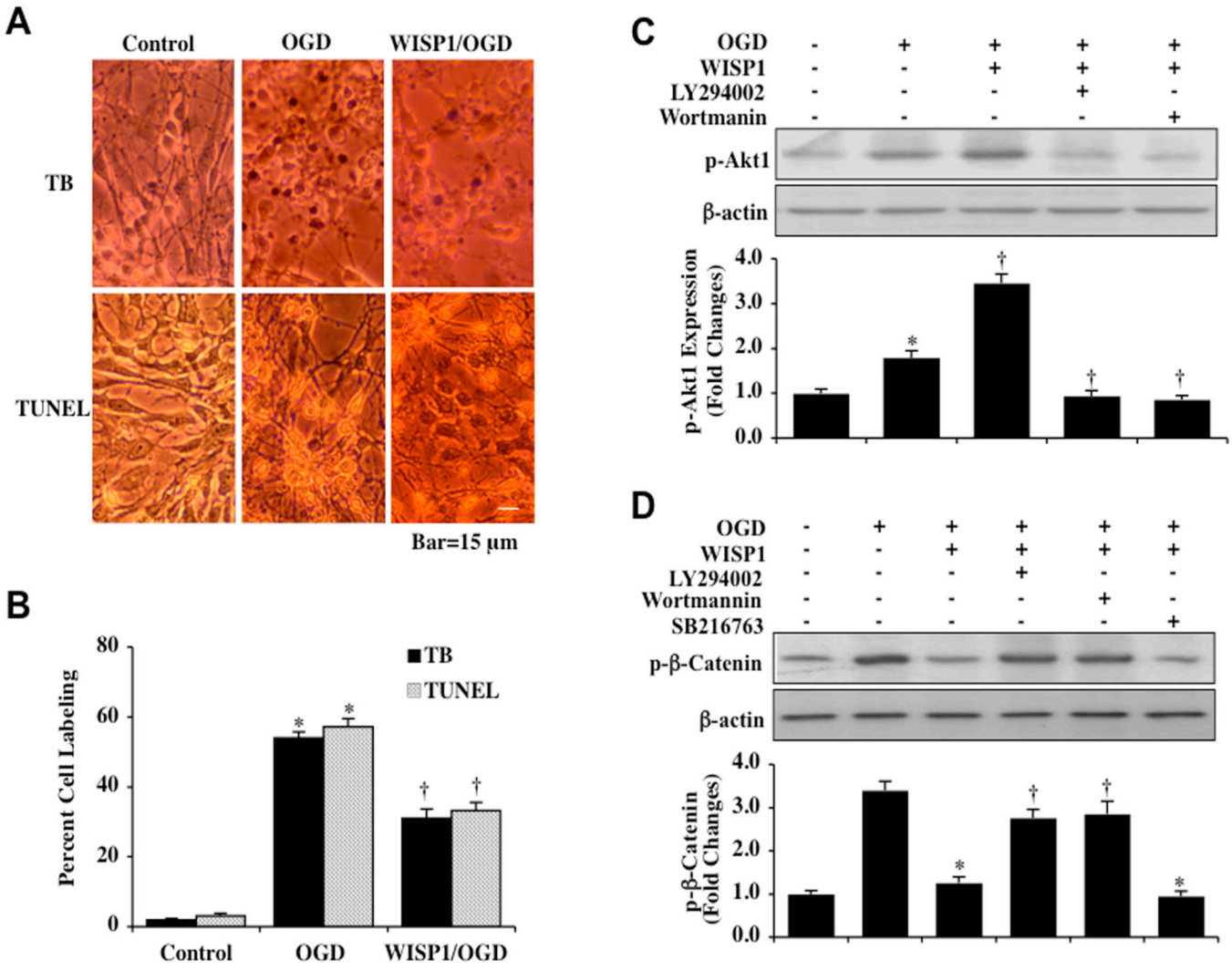

Fig. (1). WISP1 prevents neuronal cell apoptosis and controls the phosphorylation of β-catenin through the PI 3-K/Akt1 pathway during OGD

[A] Primary hippocampal neurons were exposed to OGD for 3 hours and cell injury was determined by trypan blue [TB] dye exclusion and TUNEL assay 24 hours after OGD. Representative images illustrate that OGD led to neuronal cell injury with increased trypan blue and TUNEL staining. WISP1 protein [10 ng/ml] applied to the neuronal cultures 1 hour prior to OGD significantly reduced trypan blue and TUNEL staining in neurons 24 hours following OGD. [B] Quantitative analysis shows that percent cell trypan blue staining and apoptotic DNA fragmentation was significantly increased 24 hours following OGD [$^{*}P<0.01$ *vs.* control]. In contrast, WISP1 significantly decreased percent trypan blue staining and DNA fragmentation [$^{\dagger}P < 0.01$ vs. OGD]. Each data point represents the mean and SEM from 6 experiments. [C] Equal amounts of neuronal protein extracts [50 μg/lane] were immunoblotted at 3 hours after a 3 hour period of OGD with anti–phospho-Akt1 [p-Akt1, Ser ^{473}] antibody. Administration of WISP1 [10 ng/ml] 1 hour prior to OGD significantly enhanced p-Akt1 expression. Yet, application of the specific PI 3-K inhibitors wortmannin [0.5 μM] or LY294002 [10 μM] 1 hour prior to a 3 hour period of OGD blocked expression of p-Akt1 [$^{*}P<0.01$ *vs.* Control; $^{\dagger}P<0.01$ *vs.* OGD]. Quantitative analysis of the western blots for the data from 3 experiments was performed using the public domain NIH Image program [available on the Internet at http://rsb.info.nih.gov/nih-image/]. [D] Equal

amounts of neuronal protein extracts [50 μg/lane] were immunoblotted with p-β-catenin [$Ser^{33/37}$] antibody at 3 hours following a 3 hour period of OGD. Expression of p-β-catenin was increased at 3 hours following OGD exposure and was significantly decreased by WISP1 [10 ng/ml] administration 1 hour prior to OGD. Yet, combined treatment with specific PI 3-K inhibitors wortmannin [0.5 μM] or LY294002 [LY, 10 μM] abrogated the ability of WISP1 to reduce the expression of p-β-catenin. Application of the GSK-3β inhibitor SB21673 [5 μM] 1 hour prior to OGD significantly reduced the expression of p-β-catenin 3 hours following OGD [$^{*}P<0.01$ *vs.* OGD; $^{\dagger}P<0.01$ *vs.* WISP1/OGD]. Each data point represents the mean and SEM from 3 experiments.

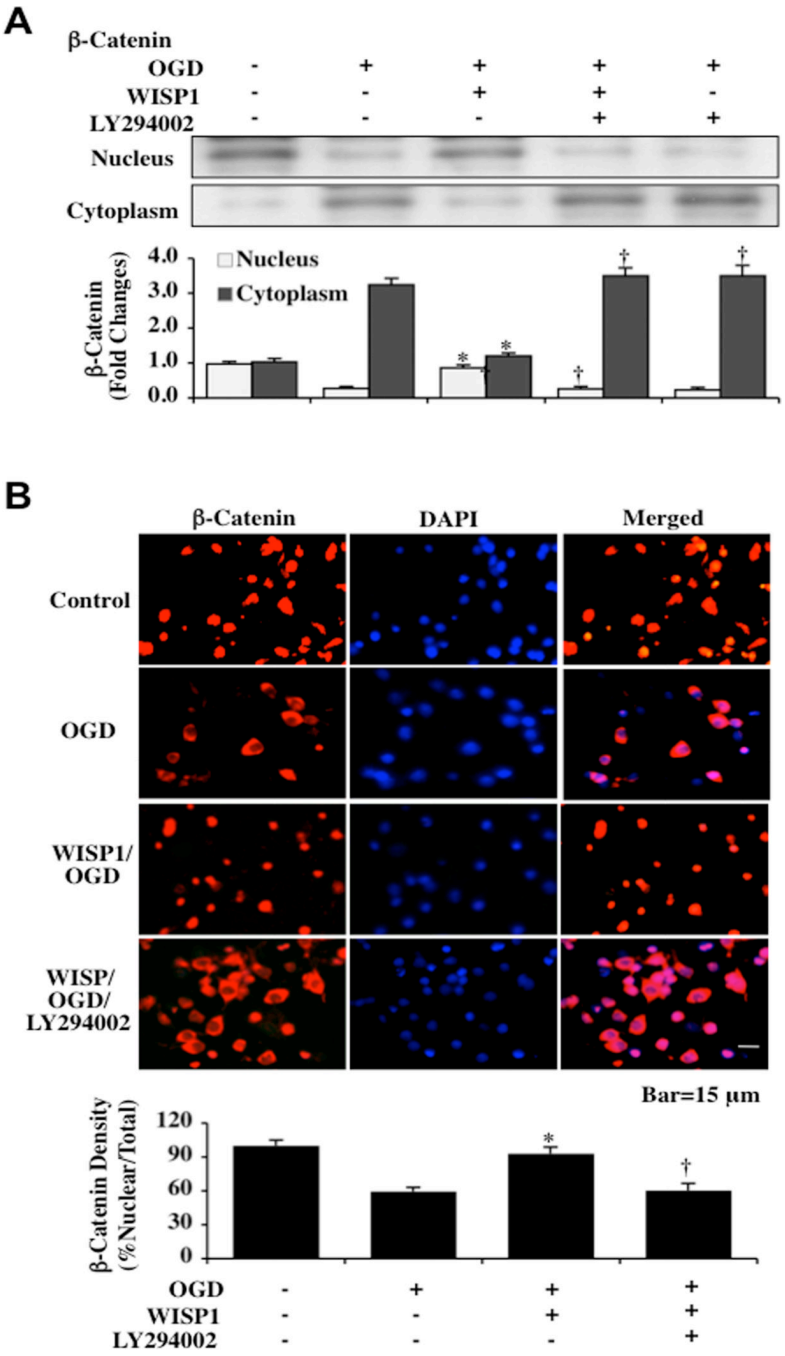

Fig. (2). WISP1 through the PI 3-K pathway fosters nuclear translocation of β-catenin during OGD

[**A**] Equal amounts of cytoplasmic [cytoplasm] or nuclear [nucleus] protein extracts were immunoblotted with anti-total β-catenin antibody at 3 hours following a 3 hour period of OGD. WISP1 [10 ng/ml] treatment significantly increased the expression of β-catenin in the nuclei of neurons. Application of the PI 3-K inhibitor LY294002 [10 μM] prevented the nuclear translocation of β-catenin during WISP1 administration and OGD exposure [*P<0.01 *vs.* OGD; †P< 0.01 vs. WISP1/OGD]. Quantitative analysis of the western blots for the data was performed using the public domain NIH Image program [available on the Internet at http://rsb.info.nih.gov/nih-image/]. [**B**] WISP1 [10 ng/ml] was administered 1

hour prior to a 3 hour period of OGD and immunofluorescence staining for β-catenin [Texas-red] was performed 3 hours after OGD. Nuclei of neurons were counterstained with DAPI. In merged images, cells with OGD alone show neuronal nuclei with minimal β-catenin staining [blue/white] with significant β-catenin staining [red] in the cytoplasm. In contrast to neurons treated with WISP1 [10 ng/ml] and OGD exposure, β-catenin is predominantly in the nuclei of neurons [nuclei primarily red]. Treatment with the PI 3-K inhibitor LY294002 [10 μM] combined with WISP1 resulted in the lost of nuclear staining of β-catenin [$^{*}P<0.01$ *vs.* OGD; $^{\dagger}P< 0.01$ *vs.* WISP1/OGD]. Intensity of β-catenin nuclear staining was performed using the public domain NIH Image program [available on the Internet at http://rsb.info.nih.gov/nih-image/] and control = untreated neurons.

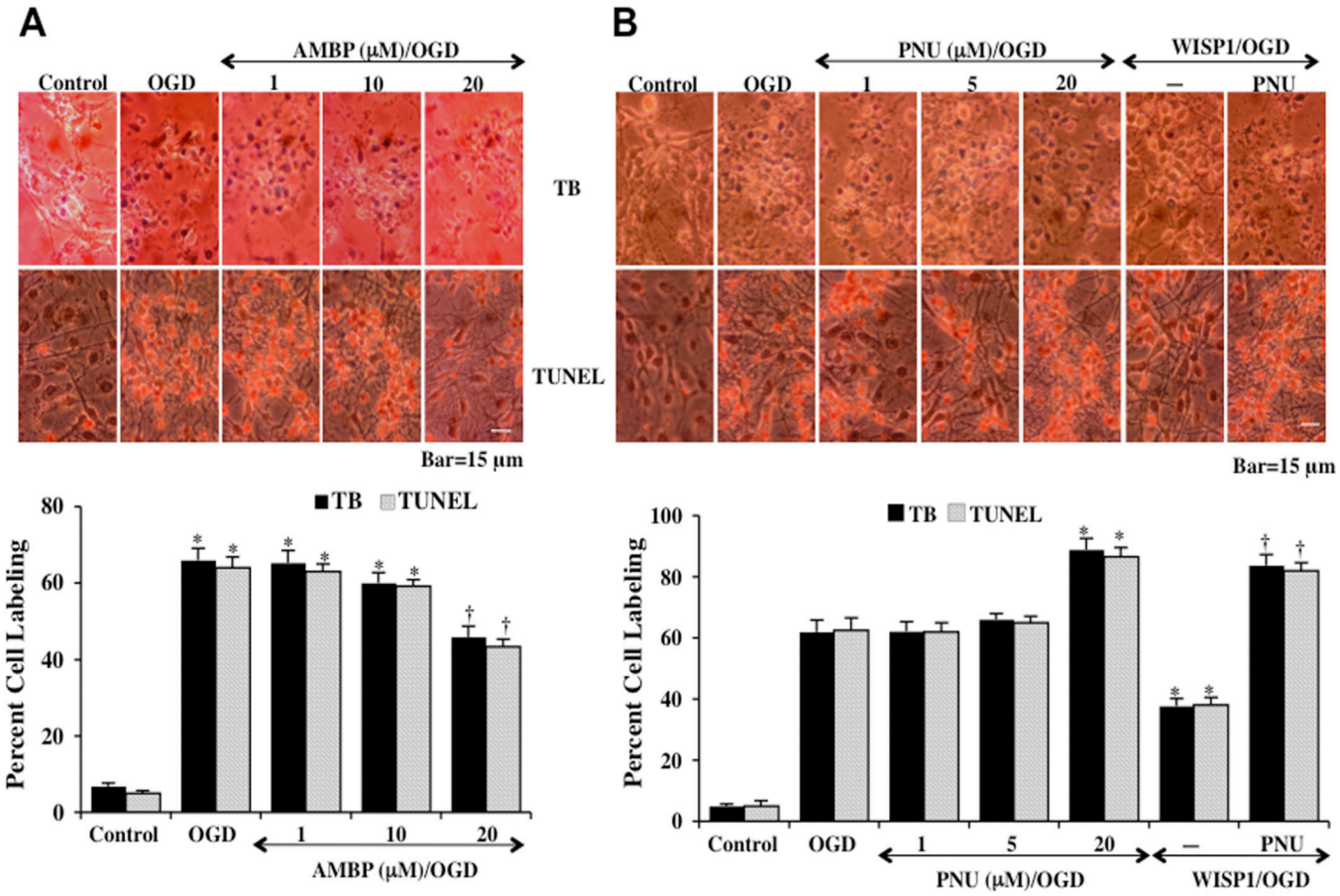

Fig. (3). WISP1 protects neurons against OGD through β-catenin activation

[A] The β-catenin agonist [AMBP, 1, 10, and 20 μM] was applied 1 hour prior to a 3 hour period of OGD. Cell injury was determined by trypan blue dye exclusion method and TUNEL staining 24 hours following OGD. Representative images demonstrate that OGD led to a significant increase in trypan blue staining and DNA fragmentation in neuronal cells 24 hours after OGD compared to untreated control cultures. AMBP [20 μM] application 1 hour prior to OGD significantly reduced neuronal cell injury. Quantitative results illustrate that AMBP [20 μM] application significantly decreased percent trypan blue uptake and DNA fragmentation 24 hours after OGD when compared to OGD exposure alone [$^{*}P<0.01$ *vs.* untreated control; $^{\dagger}P<0.01$ *vs.* OGD]. [B] The β-catenin inhibitor [PNU, 1, 5, and 20 μM] was administered 1 hour prior to a 3 hour period of OGD and cell injury was determined by trypan blue dye exclusion and TUNEL staining 24 hours following OGD. Representative images demonstrate that PUN [20 μM] increased trypan blue staining and DNA fragmentation in neuronal cells 24 hours after OGD. Combined application of the β-catenin inhibitor PNU [20 μM] with WISP1 [10 ng/ml] 1 hour prior to a 3 hour period of OGD attenuated the efficacy of WISP1 to prevent cell injury [trypan blue] and apoptosis [TUNEL] 24 hours following OGD [$^{*}P<0.01$ *vs.* OGD; $^{\dagger}P<0.01$ *vs.* WISP1/OGD]. In all cases, each data point represents the mean and SEM from 6 experiments.

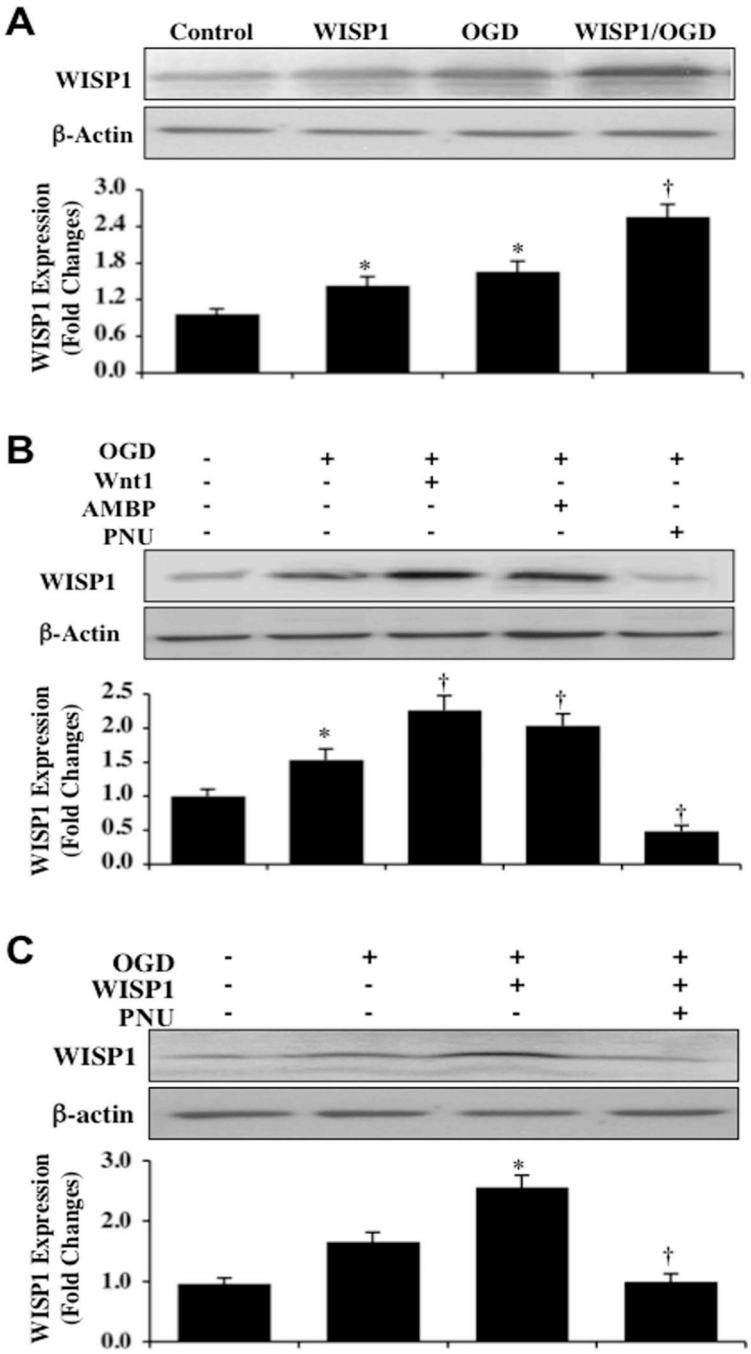

Fig. (4). WISP1 increases its expression and autoregulates its expression through β-catenin during OGD exposure

[A] Hippocampal neuronal protein extracts [50 μg/lane] were immunoblotted with anti-WISP1 antibody 24 hours following WISP1 [10 ng/ml] administration alone or following a 3 hour period of OGD with 1 hour pretreatment with WISP1 [10 ng/ml]. WISP1 treatment significantly increased WISP1 expression 24 hours both in untreated control cultures and in OGD exposed neurons [$^{*}P<0.01$ *vs.* Control; $^{\dagger}P<0.01$ *vs.* OGD]. [B] Wnt1 [100 ng/ml], β-catenin agonist [AMBP, 20 μM], or β-catenin inhibitor [PNU, 20 μM] was applied to neuronal cultures 1 hour prior to a 3 hour period of OGD and the expression of WISP1 was determined 24 hours following OGD. Both Wnt1 and AMBP treatment significantly

increased the expression of WISP1 following OGD. In contrast, PUN significantly decreased WISP1 expression following OGD [$*P<0.01$ *vs.* Control; $^{\dagger}P<0.01$ *vs.* OGD]. Control=untreated cultures. [C] Application of PNU [20 μM] with WISP1 [10 ng/ml] 1 hour prior to a 3 hour period of OGD blocked the ability of WISP1 to increase its expression 24 hours following OGD [$*P<0.01$ *vs.* OGD; $^{\dagger}P<0.01$ *vs.* WISP1/OGD]. Quantitative analysis of the western blots for the data from 3 experiments was performed using the public domain NIH Image program [available on the Internet at http://rsb.info.nih.gov/nih-image/].

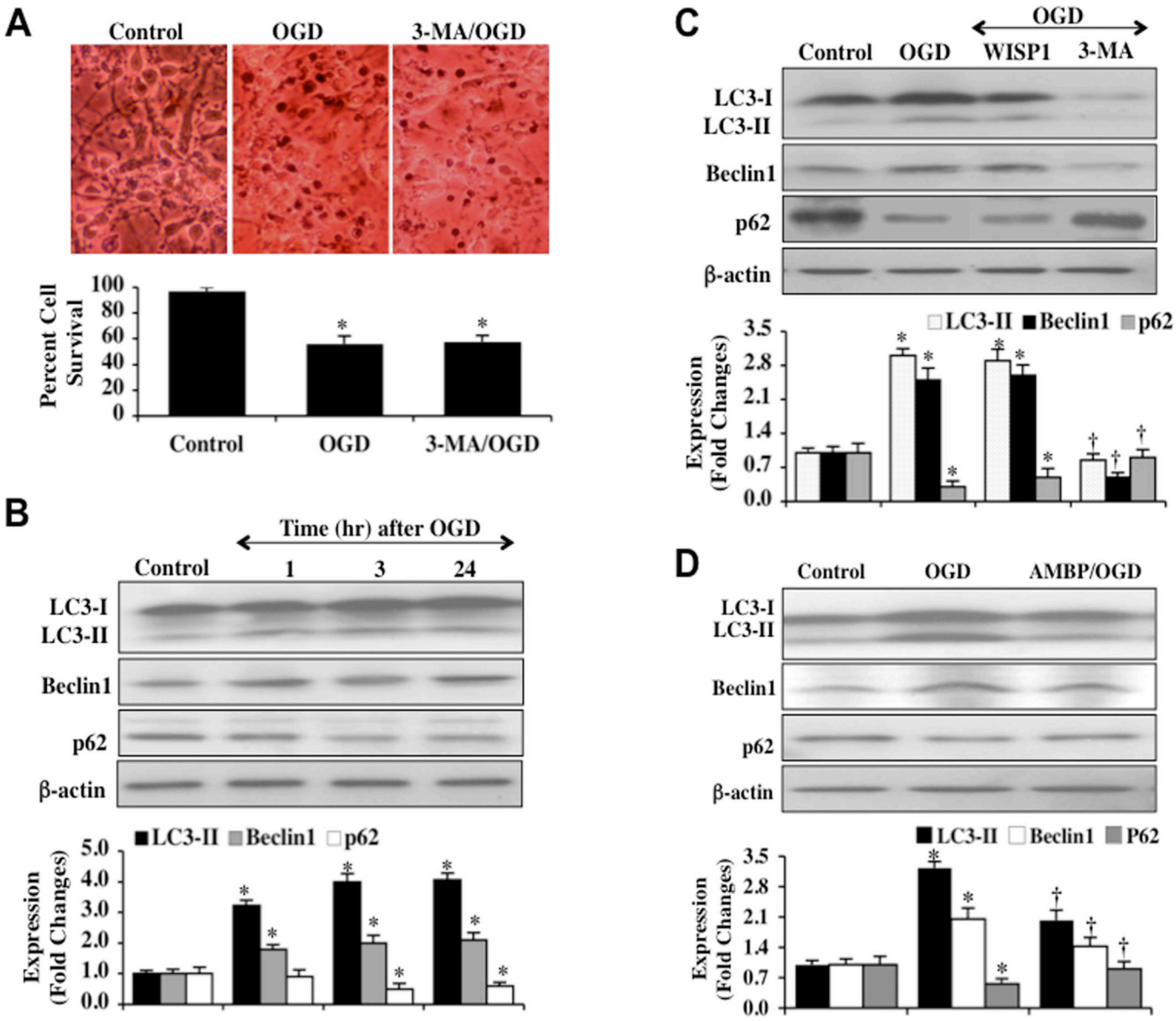

Fig. (5). Autophagy has a limited role in cell injury and WISP1 cytoprotection involving β-catenin

[A] Autophagy inhibitor 3- methyladenine [3MA, 10 mM] was applied to neuronal cultures 1 hour prior to a 3 hour period of OGD and cell survival was determined by trypan blue dye exclusion method. Representative images and quantitative results demonstrate that OGD led to a significant increase in trypan blue staining in neuronal cells at 24 hours after OGD. Inhibition of autophagy by 3MA did not improve neuronal cell survival significantly when compared with OGD treated alone neurons [**P* <0.01 *vs.* untreated Control]. Each data point represents the mean and SEM from 6 experiments. [B] Equal amounts of neuronal protein extracts [25–50 μg/lane] were immunoblotted at 1, 3 and 24 hours following a 3 hour period of OGD with anti–LC3-II, p62, and Beclin-1 antibodies. OGD resulted in a significant increase in the expression of LC3-II and Beclin-1 and a decrease in the expression of p62 [**P*<0.01 vs. Control]. [C] WISP1 [10 ng/ml] was applied to neuronal cultures 1 hour prior to a 3 hour period of OGD and the expressions of LC3-II, p62, and Beclin-1 were determined by western blot analysis 24 hours following OGD. WISP1 treatment did not

significantly change the expression of LC3-II and Beclin-1, but mildly increased p62 expression following OGD. In contrast, the autophagy inhibitor 3MA [10 mM] significantly decreased the expression of LC3-II and Beclin-1 and increased the expression of p62 at 24 hours following OGD [$^{*}P<0.01$ *vs.* Control; $^{\dagger}P<0.01$ *vs.* OGD]. [D] Application of the β-catenin agonist AMBP [20 μM] 1 hour prior to a 3 hour period of OGD decreased the expression of LC3-II and Beclin-1 and increased the expression of p62 24 hours following OGD [$^{*}P<0.01$ *vs.* Control; $^{\dagger}P<0.01$ *vs.* OGD].]. Each data point represents the mean and SEM from 3 experiments. In B, C, and D, quantitative analysis of the western blots for the data from 3 experiments was performed using the public domain NIH Image program [available on the Internet at http://rsb.info.nih.gov/nih-image/].