Int J Physiol Pathophysiol Pharmacol 2013;5(4):216-227
www.ijppp.org /ISSN:1944-8171/IJPPP1308004

Original Article

Investigating the role of the actin regulating complex ARP2/3 in rapid ischemic tolerance induced neuro-protection

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Received August 31, 2013; Accepted October 27, 2013; Epub December 15, 2013; Published December 30, 2013

Abstract: Neuronal morphology is highly sensitive to ischemia, although some re-organization may promote neuroprotection. In this study we investigate the role of actin regulating proteins (ARP2, ARP3 and WAVE-1) and their role in rapid ischemic tolerance. Using an established *in vitro* model of rapid ischemic tolerance, we show that WAVE-1 protein levels are stabilized following brief tolerance inducing ischemia (preconditioning). The stabilization appears to be due to a reduction in the ubiquitination of WAVE-1. Levels of ARP2, ARP3 and N-WASP were not affected by ischemic preconditioning. Immunocytochemical studies show a relocalization of ARP2 and ARP3 proteins in neurons following preconditioning ischemia, as well as a re-organization of actin. Blocking the protein kinase CK2 using emodin blocks ischemic tolerance, and our data suggests CK2 binds to WAVE-1 in neurons. We observe an increase in binding of the ARP2 subunit with WAVE-1. The neuroprotection observed following preconditioning is inhibited when cells are transduced with an N-WASP CA domain that blocks the activation of ARP2/3. Together these data show that ischemia affects actin regulating enzymes, and that the ARP2/3 pathway plays a role in rapid ischemic tolerance induced neuroprotection.

Keywords: Actin, WAVE-1, ischemic tolerance, neurons, Arp2/3

Introduction

Multiple studies show that neuron morphology is rapidly changed following exposure to ischemic conditions. While originally thought to be due to excitotoxicity, it has also been shown that sub-toxic ischemic exposures result in a similar, albeit, transient morphological reorganization of dendritic morphology [1-3]. The reorganization of dendritic morphology plays a role in neuroprotection and resilience to normally toxic concentrations of the glutamate agonist NMDA (N-Methyl-D-Aspartate) [4]. Reorganization of dendritic morphology was recently described in the acquired protective state of rapid ischemic tolerance [4]. Rapid ischemic tolerance is the phenomenon, whereby exposure to non-toxic ischemic stress promotes a transient period of tolerance to subsequent normally harmful ischemia 30-60 min following the preconditioning event [4]. The remodeling of dendritic spines following ischemia is likely due to glutamate release, since incubation of neurons with glutamate provokes a rapid loss of dendritic spines, and the formation of varicosities [3, 5, 6]. Interestingly, both ischemic and excitotoxic induced changes in spine morphology and number are blocked by proteasome inhibitors, such as MG132 (aka carbobenzoxy-Leu-Leu-Leucinal) [4, 6].

The actin cytoskeleton has many important cellular functions, and pertinent to this study is critical for the formation of dendritic spines. Spines are the site of synaptic contact and contain complex signaling structures at the post synaptic density, which are anchored to the actin cytoskeleton via actin binding scaffold proteins. Actin forms branched filaments in the spine, and the branching (nucleation) of actin

has been shown to be regulated by the ARP2/3 complex of proteins [7, 8]. Actin nucleation is mediated by actin-related protein 2 (ARP2) and actin-related protein 3 (ARP3) subunits of the complex, and the remaining subunits help to position these catalytic proteins at the point of nucleation. The stoichiometry of ARP complex proteins appears to be 1:1 with each other [8]. Interestingly, humans and mice have two isoforms of ARP3 [9], and RNAi-mediated knockout of ARP3 promotes cell death in human cells [10], but not mice [11]. Activity of the ARP2/3 complex is inhibited by multiple proteins, such as the synaptic Protein Interacting with C Kinase (PICK1) [12]. The ARP2/3 complex is activated by multiple proteins, of which the most common are neural Wiskott-Aldrich syndrome protein (N-WASP) and Wasp family verprolin homologous protein (WAVE: aka suppressor of cAMP receptor protein: SCAR) [13-16]. These proteins are activated by phosphorylation of a carboxyl-terminus regulatory VCA domain, which then binds to ARP2/3 proteins to promote nucleation [15] (see **Figure 5** for overview). Additional activators of the ARP2/3 complex have also been described including WASP and SCAR Homologue (WASH) and the junction mediating and regulatory protein (JMY) (see [17]).

Our interest in the ARP2/3 signaling complex came from two observations. First, our previous studies showing re-organization of actin in the neuron following brief ischemic stress [4]. Visualization of actin using phalloidin suggests a filamentous staining pattern following preconditioning ischemic stress, however the ratio of F-actin/G-actin is reduced in these cells [4]. Second, from our proteomic study of ubiquitinated protein following brief ischemic stress, we observed both WAVE-1 and its inhibitory protein CYFIP both showing a reduction in ubiquitination following brief ischemic stress [4]. This led us to hypothesize that WAVE-1 and ultimately the ARP2/3 complex may play a role in ischemic tolerance. Therefore we investigated whether the reduction of ubiquitination of WAVE-1 following preconditioning ischemia was associated with a change in protein levels, whether WAVE-1 was binding to and activating the AR2/3 complex following preconditioning ischemia, and whether the ARP2/3 complex played a role in rapid ischemic tolerance (See **Figure 5** for overview).

Methods and materials

Reagents

The proteasome inhibitor MG-132 (Z-Leu-Leu-Leu-CHO) was purchased from Calbiochem, (San Diego, CA). The following antibodies were used; WAVE-1 (AB4095), N-WASP (AB1963) ARP2 (AB3886) (Chemicon), CYFIP (07-531), ARP3 (07-531) (Upstate), (alpha-Tubulin SC80-35) CK2 (SC12738) (Santa Cruz Biochemicals), VCA-Agarose (Cytoskeleton Inc.); p62-UBA agarose beads (ENZO life Sciences). VCA viral vectors and GFP control vectors were a kind gift from Dr Lorene Lanier (U. Minnesota).

Cell culture

Animal procedures were performed at Legacy Research, Portland, OR, or at Morehouse School of Medicine, Atlanta, GA, at facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and in accordance with protocols approved by the respective IACUCs, as well as the principles outlined in the National Institute of Health *Guide for the Care and Use of Laboratory Animals.* Primary rat neuronal cultures were prepared as described previously (Meller et al., 2008). Briefly cortices were removed from one day old rat pups (mixed sex) incubated in papain (Worthington Biochemicals, NJ, US) and then triturated with a fire polished glass pipette. Neurons were grown for 14 days in Neurobasal A medium supplemented with B27 (Invitrogen, Carlsbad US) and Neurocult supplement (Stem Cell Technologies, Vancouver BC, Canada).

Oxygen and glucose deprivation modeled ischemia

Oxygen and glucose deprivation (OGD) was used to model ischemia, as previously described (Meller et al., 2005; Meller et al., 2006). Cells were washed twice with phosphate buffered saline solution supplemented with magnesium and calcium (NaCl 1.37 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.7 mM, $CaCl_2$, 0.5 mM, $MgCl_2$ 1 mM, pH 7.4), then placed in 1.5 ml PBS solution and transferred to an anoxic chamber (85% N_2, 5% H_2, 10% CO_2; 35 °C) (Bactron or Forma Scientific) for indicated periods of time. To terminate ischemia, cells were replenished with Neurobasal A media and returned to a normoxic chamber.

Immunoblotting

Immunoblotting was performed as previously described [18]. Tissue samples were lysed in a non-denaturing buffer containing the protease inhibitors phenylmethylsulfonylfluoride (100 µg/ml), aprotinin (1 µg/ml), leupeptin (1 µg/ml), pepstatin (1 µg/ml), NaF (50 mM) Na_3VO_4 (2 mM) and phosphatase inhibitor cocktail (Sigma, St Louis, MO). Protein concentration was determined by Bradford reagent spectrophotometrically at A_{595}. Protein samples (50 µg) were denatured in a gel-loading buffer at 100 °C for 5 min and then loaded onto 12% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes and incubated with primary antibodies at 4 °C overnight. Membranes were incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Cell Signaling Technology, Beverly, MA, USA) followed by chemiluminescence detection (Visualizer, Millipore) for 2 minutes prior to digital determination of luminescence (Kodak 200 RT or 4000 MM image station). Chemiluminescence was quantified using the Kodak 1D software.

Pulldown and immunoprecipitation

Cells were plated out on 10 cm dishes at 8 million cells/dish. Tissue samples were lysed in a nondenaturing buffer containing the protease inhibitors phenylmethylsulfonylfluoride (100 µg/ml), aprotinin (1 µg/ml), leupeptin (1 µg/ml), pepstatin (1 µg/ml), NaF (50 mM) Na_3VO_4 (2 mM) and phosphatase inhibitor cocktail (Sigma, St Louis, MO). Total protein (250-500 µg) was cleared and then incubated with either 30µl P62 UBA agarose beads (Affiniti, Exeter, UK) at 4 °C for 2 hours, VCA-agarose beads (Cytoskeleton) at 4 °C for 2 hours or 1-10 µg of primary antibody overnight at 4 °C. Antibody bound proteins were precipitated using agarose-A beads and all samples were washed using an immunoprecipitation kit (IP50, Sigma). The protein/bead mixture was denatured at 95 °C for 5 min and then loaded onto 10% SDS-polyacrylamide gels. Blots were subject to immunoblotting as above. Ubiquitin pull down was verified by re-probing blots with anti-ubiquitin antibody (Santa Cruz, CA).

Immunocytochemistry

Cultured cortical neurons of 10-14 DIV treated with or without OGD preconditioning were fixed with 5% neutral buffered formalin (Sigma) for 30 min at room temperature, permeabilized with PBS containing 0.01% Triton X-100 at 4 °C for 1-2 hours, and incubated with antibodies specific to ARP2 (Chemicon), ARP3 (Upstate) and WAVE-1(Chemicon) at 4 °C overnight. After washing with PBS containing 0.01% Triton X-100, secondary antibodies conjugated to AlexaFluor 488 (Invitrogen) were applied at room temperature for 2 hours to detect primary antibodies. Coverslips were then washed again and mounted with Fluoroshield (Sigma) or VectaShield (Vector Labs) containing DAPI. Slides were allowed to dry overnight at 4 °C in dark before imaging. Immunostaining was imaged using a Carl Zeiss Axioimager M2 fitted with EC Plan Neo-Fluar (40×) and Plan Apochromat (100× oil) objectives under Ex/Em wavelengths of 359/461 nm (blue) and 470/509 nm (green). Images were captured using a Zeiss MRM camera and analyzed using Axiovision v4.6 software. Arp2/3 and Actin co-localization images were subjected to medium deconvolution using the nearest-neighbors algorithm in Zeiss Axiovision.

Cell death experiments

Four sets of experimental conditions were investigated in the ischemic tolerance paradigm. Cells received either control wash only, 30 min OGD, 120 minute OGD, or 30 min OGD followed by 1 hour recovery in normoxic conditions with Neurobasal A media followed by 120 min OGD. Cells are left to recover for 24 hours following the final wash or ischemic event and cell death is assessed by propidium iodide (PI) exclusion assay [4]. Cells are fixed, counter stained with DAPI and the number of PI positive neurons counted. Coverslips are imaged at 3 random areas, and the number of DAPI positive and PI positive cells determined using Image J.

Results

WAVE-1 expression in an in vitro model of rapid ischemic tolerance

These studies are based on our cell culture model of rapid ischemic tolerance [19]. Rat cortical neurons, of 10 days in vitro, are subjected to ischemia, modeled using oxygen and glucose deprivation (OGD) (**Figure 1A**). Consistent with our previous studies, we find low basal cell death in primary cultures of rat cortical neurons (typically approx. 90% neurons). A precon-

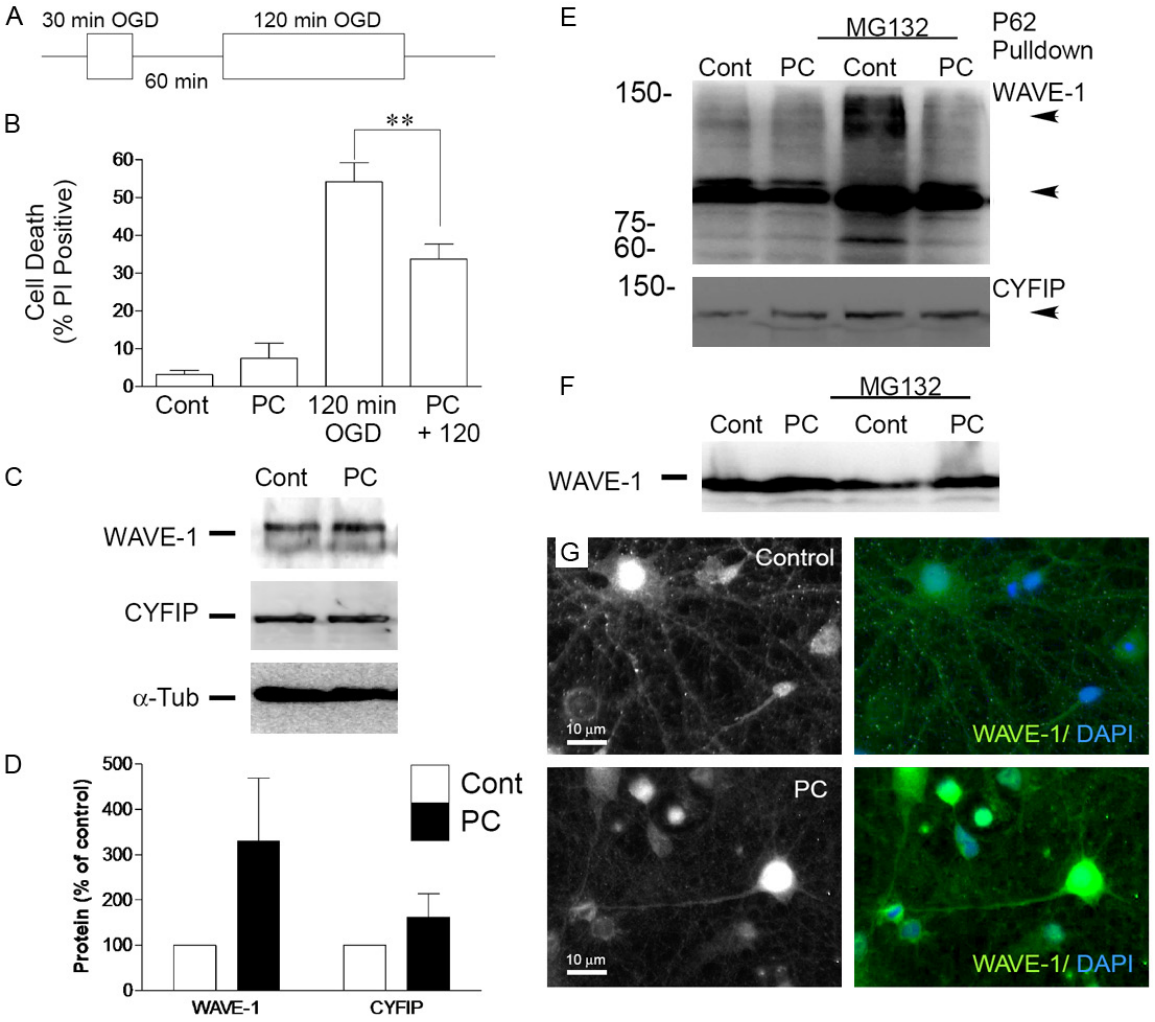

Figure 1. A. Schematic of ischemic tolerance paradigm. A 30 minutes oxygen and glucose deprivation is paired with 120 min OGD 1 hour later. Cell death is assessed 24 h later using propidium iodide staining. B. Analysis of cell death in ischemic tolerance model, data shown are mean ± sem of n=8 observations, **denotes P<0.01. C. Immunoblot analysis of proteins from cortical neurons subjected to 30 min ischemia and recovered for 1 h. D. Quantification of 4 experiments, data shown are mean ± sem of n=4. E. Wave-1 ubiquitination revealed by pulldown with an immobilized ubiquitin binding domain form P62. Cells were incubated with P62 beads for 1 h, washed and then wave-1 detected using immunoblotting. Some cells were also incubated with MG132 in the recovery period. F. Wave-1 protein levels IN ischemic preconditioning treated cells and in cells incubated with MG132. Representative blot of 3 experiments. G. Immunocytochemistry of WAVE-1 in cultured neurons following preconditioning ischemia. Cells were subjected to 30 min OGD, recovered for 1 h and then fixed. Wave-1 staining was detected with an Alexa-fluor-488 conjugated secondary antibody and coverslips were counterstained with DAPI. Images were obtained with a 40× objective. Representative image of 2 experiments. Scale bar = 10 μm.

ditioning duration of 30 min OGD does not significantly affect cell death (**Figure 1B**). In contrast a 120 min duration of OGD results in 50-60% cell death, as determined by PI exclusion assay (P<0.001) (**Figure 1B**). When neurons are exposed to a 30 min OGD one hour prior to the 120 min OGD we observe a 50% reduction in cell death compared to non-preconditioned cells subjected to 120 min OGD (54.1±5.1 vs. 33.7±4.0% cell death P<0.01) (**Figure 1B**). Since neuroprotection occurs one hour following the preconditioning event, we focused our biochemical analysis on this time point.

Based on our previous study we initially focused our work on measuring the levels of WAVE-1 and CYFIP (also known as PIR121) in neuronal lysates following preconditioning ischemia. Our previous study suggested that the ubiquitina-

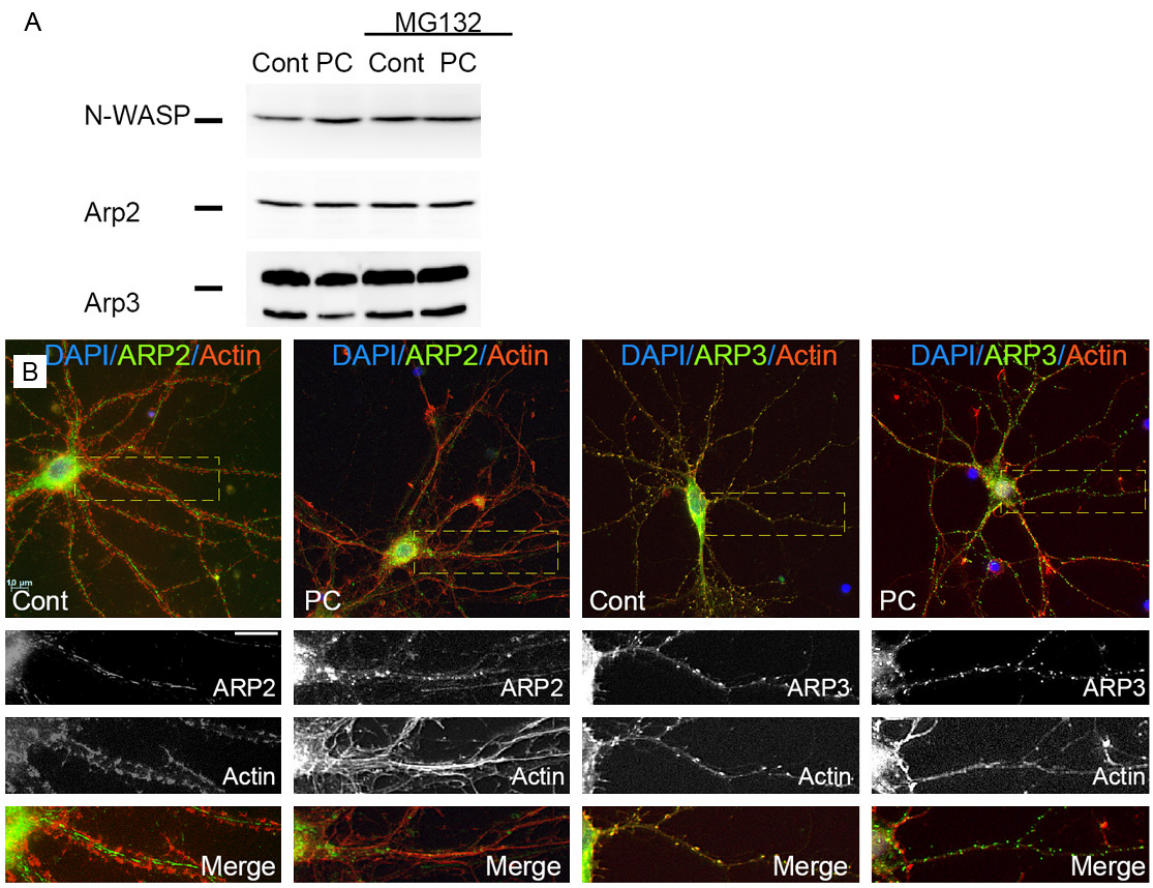

Figure 2. A. Immunoblotting of N-WASP, ARP2, and ARP3 in neuronal cultures subjected to ischemic preconditioning. B. Representative images of neuronal cultures stained for ARP2 and ARP3 in control and ischemic preconditioned cells. Cells were stained with Phalloidin-546 to visualize actin. Scale bar = 10 μm; hashed box denotes enlarged area. Images were subjected to nearest neighbor deconvolution.

tion of both WAVE-1 and CYFIP proteins was reduced following preconditioning ischemia [4]. WAVE-1 was expressed as an 84 kDa protein. One hour following preconditioning ischemia protein levels increased approximately three fold (**Figure 1C** and **1D**). CYFIP inhibits the activation of WAVE-1, but also plays a role in regulating the activity of fragile X mental retardation protein [20]. CYFIP was expressed as a 120 kDa protein. In contrast to WAVE-1, we saw a 1.5 fold increase in CYFIP levels following preconditioning ischemia (**Figure 1C** and **1D**).

We further investigated whether changes in protein levels were due to protein ubiquitination using a P62 pulldown assay [4]. In control cells, WAVE-1 pulldown was higher than the weight of the endogenous protein (approximately 18-20 kDa). P62 pulldown of the higher weight WAVE-1 was reduced in preconditioning-treated cells (**Figure 1E**). To confirm that the species we measure is related to ubiquitination of WAVE-1 we incubated some cells with the proteasome inhibitor MG132 (1.0 μM, 1 h). Ubiquitination of WAVE-1 was enhanced in the presence MG132 and showed higher molecular weight smearing that is indicative of ubiquitination (**Figure 1E**). The high weight ubiquitination of WAVE-1 was reduced in preconditioned cells treated with MG132 (**Figure 1E**).

Finally, we investigated WAVE-1 protein levels in cells treated with MG132. In control cells treated with MG132 we observe a reduction in native weight WAVE-1. This reduction in WAVE-1 levels is reversed by preconditioning ischemia (**Figure 1F**). These observations are consistent with our mass spectrometry study [4], in which cells were treated with MG132 following preconditioning ischemia to stabilize the ubiquitinated protein products. This suggests that WAVE-1 is under tonic regulation by the ubiquitin proteasome system (UPS) and that precon-

ditioning ischemia reduces its UPS-mediated degradation.

We then investigated the ubiquitination of CYFIP. In control cells, CYFIP pulldown was at the normal weight for CYFIP (121 kDa) in addition no high molecular weight smear was evident under control or MG132 treated cells (**Figure 1E**). These data lead us to conclude that in this system CYFIP ubiquitination is not subject to ischemia-mediated regulation. Hence we focused our investigation on WAVE-1.

We investigated the cellular localization of WAVE-1 using ICC. We observed WAVE-1 in both the nucleus of neurons (**Figure 1G**), and with a strong dendritic staining pattern similar to that previously reported [21, 22]. This pattern is similar to that observed with actin staining in control neurons (See **Figure 2B**). Following preconditioning the nuclear and perinuclear staining was enhanced, and the dendritic staining was less pronounced, but appeared similar to the staining of actin in neurons following preconditioning ischemia. (**Figure 1G**). Some staining was evident in simple looking dendrites, which corresponds to the observed changes in neuronal morphology following preconditioning ischemia [4]. Together these data suggest that WAVE-1 relocates in the neuron following preconditioning stress.

ARP2/3 protein levels in an in vitro model of rapid ischemic tolerance

WAVE-1 has been shown to regulate dendritic spine morphology via its interaction with the ARP2/3 complex. Actin reorganization is a hallmark cytological feature of rapid ischemic tolerance (See **Figure 2B**), therefore we focused our investigation on the ARP2/3 complex and its activating enzymes using immunoblotting following 30 min OGD. We did not observe a significant change in protein levels of N-WASP and ARP2 following preconditioning ischemia, in contrast, ARP3 levels were slightly reduced (approx. 40%) (**Figure 2A**). There was no effect of incubating cells with MG132 on the protein levels of N-WASP, ARP2 or ARP3 (**Figure 2A**).

Localization of ARP2, ARP3 following preconditioning ischemia

We investigated whether preconditioning ischemia resulted in changes to the localization of the ARP2/3 complex associated proteins and actin. Actin was visualized using Alexafluor 546 labeled phalloidin. Similar to our previous study, we observe changes in actin staining that are consistent with a reorganization of actin in the cell and a change in neuronal morphology following brief ischemic stress. In control cells actin has a more punctate staining pattern in dendrites, which correlates to the localization of spines and spine heads. In contrast following preconditioning ischemia actin relocalizes to the dendritic shaft of the neuron. In addition, following preconditioning ischemia there was a stronger phalloidin staining signal in the nucleus (**Figure 2B**).

Immunocytochemical analysis reveals a perinuclear staining pattern of ARP2 in control neurons. ARP2 staining is also observed in dendrites where it co-localizes with actin (**Figure 2B**). The dendrites show a punctate actin staining pattern. In control cells peri-nuclear staining for ARP2 is observed. Following preconditioning ischemic stress the nuclear staining for ARP2 increased (**Figure 2B**). Following preconditioning ischemia, there was a change in ARP2 staining, which appears related to the change in neuronal morphology. Interestingly, the ARP2 did not appear to strongly co-localize with actin in dendrites (**Figure 2B**).

Immunocytochemical analysis of ARP3 reveals a peri-nuclear staining pattern in control cells. Most of the staining for ARP3 appeared localized to dendritic spines and actin localized with spines. Some nuclear staining was also present (**Figure 2B**). Following preconditioning ischemia, we observe a loss of spine associated actin, but an increase of actin in the dendritic shaft. The ARP3 signal showed a weaker co-localization with actin following preconditioning ischemia (**Figure 2B**). These observations is similar to our previous observations of actin staining patterns in neurons [4]. These data suggest that ARP2 and ARP3 show a relocalization in cells following preconditioning ischemia. However, since the colocalization of ARP subunits with actin is lower following preconditioning ischemia, some of the relocalization may not be associated with the reorganization of actin into the dendritic shaft of neurons following preconditioning ischemia.

Interaction of WAVE-1, N-WASP and VCA domains with the ARP2/3 complex

Both WAVE-1 and NWASP are phosphorylated on the VCA domain and activated by CK2 (for-

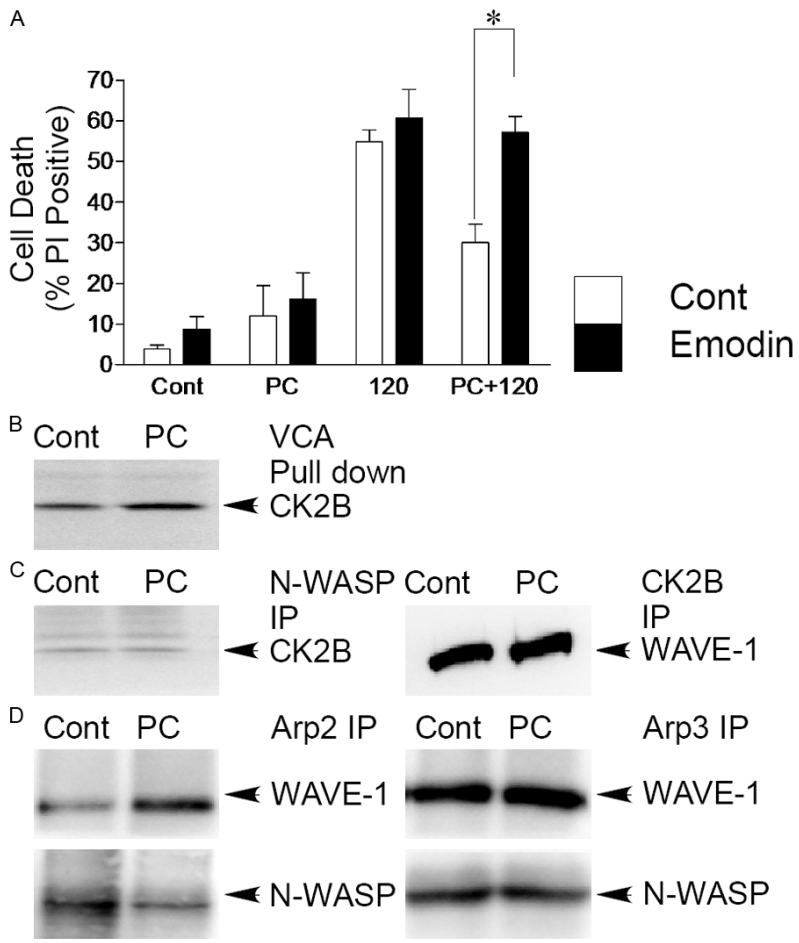

Figure 3. Emodin blocks rapid ischemic tolerance. A. Cells were preconditioned and then incubated with emodin for 1 h, cells were then subjected to 120 min OGD. Cell death was determined using PI staining. Data shown are mean ± Sem, n=4 observations, *denote P<0.01 effect of Emodin PC+120 vs. non treated PC+120 cells. B. Pulldown of CK2 with immobilized VCA domain in control and preconditioned cells. C. Immunoprecipitation of N-WASP from control and preconditioned cells blotted for CK2B. Immunoprecipitation of CK2B from control and preconditioned cells blotted for WAVE-1. D. Immunoprecipitation of Arp2 and Arp3 from control and cultures subjected to preconditioning ischemia blotted for N-WASP and WAVE-1. Images are representative blots from 2-3 3 experiments.

merly Caesin kinase II) [15, 23, 24]. We first investigated, the potential regulation of rapid ischemic tolerance using the CK2 inhibitor Emodin. Emodin did not have any significant effect on basal cell death, cell death following 30 min OGD, or 120 min OGD-induced cell death. However, the reduction in cell death observed when neurons are preconditioned one hour prior to harmful ischemia was lost when emodin was incubated with the cells for one hour following the preconditioning event (**Figure 3A**) (33.7±4.0% cell death in protected cells vs. 57.2±3.9% in emodin-treated cells P<0.01). A similar, but non-significant effect was observed with a second CK2 inhibitor DMAT (not shown).

We investigated whether CK2 was regulating N-WASP or WAVE-1 via an interaction with the VCA domain. We utilized an immobilized N-WASP/WAVE-1 VCA domain to investigate VCA CK2 interactions. We observed a small increase in CK2 pulldown with the VCA domain (**Figure 3B**). If N-WASP is being phosphorylated by CK2, then we hypothesized that we would observe an increased interaction between CK2 and N-WASP in preconditioned cells. As can be seen in **Figure 2D**, we did not observe any change in N-WASP CK2 binding following preconditioning ischemia. Therefore we investigated whether CK2 was increasing its interaction with WAVE-1. The binding of WAVE-1 and CK2 was more robust that the N-WASP CK2 interaction under basal conditions. As can be seen in **Figure 3C** there was an increase in WAVE-1 binding to CK2 following preconditioning ischemia. These data suggest that CK2 plays a role in rapid ischemic tolerance and may preferentially interact with WAVE-1 following preconditioning ischemia.

We performed further immunoprecipitation experiments between ARP2 or ARP3 and N-WASP or WAVE-1. The WAVE-1 interaction with ARP2 increased following preconditioning ischemia (**Figure 3D**), in contrast the N-WASP interaction with ARP2 was reduced following preconditioning ischemia (**Figure 3D**). The binding of ARP3 with WAVE-1 and N-WASP did not appear to change following preconditioning ischemia (**Figure 3D**).

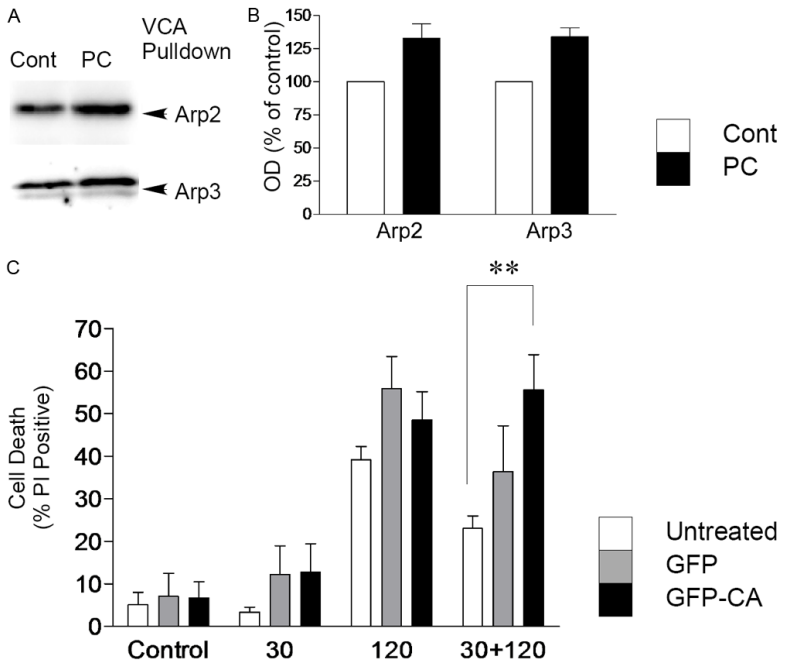

Figure 4. A. Pulldown of ARP2 and ARP3 from control and preconditioned neurons using immobilized VCA domain. B. quantification of data, mean ± sem n=4. C. Transduction of neurons with CA domain blocks rapid ischemic tolerance. Cells were incubated with virus particles for 24 h at MOI of 5, then subjected to a rapid ischemic tolerance paradigm. As a control CFP or non-transduced cells were used. Data shown are mean ± sem (n=5) **denotes P<0.01 GFP-CA particle treated cells 30+120 vs untreated 30+120 cells.

These data were further confirmed by VCA pull down experiments using the immobilized VCA domain. As can be seen in **Figure 4A**, there is a small yet consistent increase in both ARP2 binding to the VCA domain and ARP3 binding to the VCA domain following preconditioning ischemia (P<0.05). Together these data suggest that following preconditioning ischemia the stabilization of WAVE-1 results in an enhanced interaction with ARP2/3.

ARP2/3 inhibitory peptide block rapid ischemic tolerance

Finally, in order to assess the potential role of the Arp2/3 complex in rapid ischemic tolerance, ARP inhibitory peptides were tested in a model of rapid ischemic tolerance. The CA domain of N-WASP was transduced in to cells using an adenovirus vector system. Cultures were incubated with viral particles at a MOI of 5 for 48 hours. Cells were then subject to our ischemic tolerance paradigm (see above). Cells receive either, no ischemia, 30 min OGD, 120 min OGD, or 30 min OGD followed by 120 min OGD one hour later. Cells were incubated with either no virus, GFP virus or CA-GFP virus. In untreated cells, harmful ischemia induced approximately 40% cell death in the cultures, whereas cell death was not increase by the preconditioning 30 min OGD. Preconditioning the cells with 30 min OGD prior to the harmful ischemia reduced cell death by 50% (P<0.01). Incubating the cells with the CA-GFP construct resulted in a blocking of the protection compared to untreated cells (P<0.01). Interestingly greater cell death was observed following 30 and 120 min OGD, but this did not reach significance (P>0.05). Incubating cells with the GFP construct did not significantly affect ability of the cells to become tolerant to ischemia. Of note, the 30 min OGD and 120 min OGD-induced cell death rate was higher in the GFP only treated cells, but again this was not significant. Therefore, these data suggests that blocking ARP2/3 with CA-GFP peptides results in a blockade of rapid ischemic tolerance induced by preconditioning with 30 min OGD.

Discussion

In this study we investigated the effect of brief preconditioning ischemia on WAVE-1 and the ARP2/3 complex. We show that following preconditioning ischemia, WAVE-1 protein levels increase due to a reduction in its ubiquitination. Levels of N-wasp, a related protein did not change. While Arp2 and ARP3 levels were not significantly affected, the proteins relocalize in the cell following preconditioning ischemia. Our data suggest a role of CK2 in regulating the interaction of WAVE-1 with ARP complex, and finally we show that blocking Arp2/3 using a transduced CA domain from N-wasp also blocks rapid ischemic tolerance. Taken together these data suggest that WAVE-1 mediated ARP2/3 activation mediates ischemic tolerance.

We have previously reported a reduction in WAVE-1 pulldown with a P62 ubiquitin binding

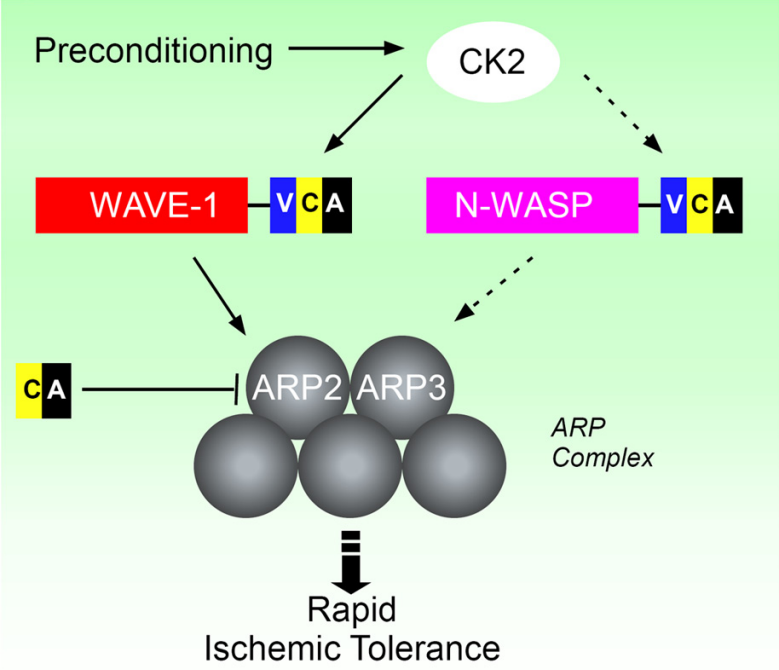

Figure 5. Overview of Hypothesis.

domain following 30 min OGD, indicating that WAVE-1 shows reduced ubiquitinylation following preconditioning (Meller et al., 2008). This finding suggested that the interaction between ARP2/3 and its activators may be altered following 30 min OGD. We followed up this observation with immunoblotting, immunocytochemistry and ubiquitin pulldown studies. Together these data show that WAVE-1 is stabilized following preconditioning ischemia. Our previous studies have reported, or focused on proteins whose ubiquitination was enhanced following preconditioning ischemia [4, 19]. Hence this unusual situation suggest that some proteins are either not ubiquitinated, or are actively deubiquitinated following preconditioning ischemia.

We then focused on other ARP2/3 complex associated protein, namely N-WASP Arp2 and Arp3. We did observe a small decrease in Arp3 compared to Arp2, which was unexpected since many studies suggest that Arp2 and Arp3 are present in the ARP2/3 complex in a 1:1 ratio. We then focused on potential upstream regulators of WAVE-1 and N-WASP. Phosphorylation of the VCA domain of N-Wasp by CK2 (formerly Caesin kinase II) increases actin polymerization by Arp2/3 [15]. The CK2 inhibitor emodin blocked rapid tolerance, and to a lesser extent the CK2 inhibitor DMAT reduced preconditioning-induced neuroprotection. However, we saw no consistent evidence that CK2 was interacting with N-WASP and driving the reaction. Since we observed an increase in CK2 binding to an immobilized VCA domain, we investigated WAVE-1. Our data show WAVE-1 interacts with CK2 following preconditioning ischemia, and increases its interaction with ARP2 following preconditioning ischemia. These data suggest that while CK2 is active and required for tolerance, it does not mediate this effect via N-WASP but rather via WAVE-1.

WAVE-1 is activated by its release from a number of regulating proteins (Abi, CYFIP etc) we did not observe changes in neither CYFIP levels, nor Abi (other proteins were undetectable with antibodies). WAVE proteins are regulated by multiple protein kinases including protein kinase A, p42/p44 MAPK and CK2. p42/p44 MAPK phosphorylates WAVE and enhances its interaction with ARP2/3, but reduces actin polymerization [14]. We have previously shown that blocking p42/p44 MAPK activation using MEK inhibitors (PD98059 and U0126) blocks rapid ischemic tolerance [19]. WAVE-2 has multiple CK2 phosphorylation sites in its VCA domain [24], which may explain, at least in part, the effects of emodin in blocking rapid ischemic tolerance. Our data supports a role for CK2 in regulating WAVE-1 in neuronal cultures following ischemia. Together it is clear that WAVE-1 regulation is via multiple complex kinase signaling cascades, and these require further elucidation in rapid ischemic tolerance.

To further investigate whether ARP2/3 availability is altered in preconditioning, the N-WASP/WAVE-1 VCA-domain conjugated to agarose was used to pulldown ARP2/3 from neuronal cell lysates. Following preconditioning ischemia (30 min OGD), there was a significant increase in the pulldown of both ARP2 and ARP3 proteins compared to controls without

OGD (**Figure 4A**, **4B**). Both N-WASP and WAVE-1 contain a Carboxyl terminus Verprolin homology domain, or VCA domain. The V sub domain binds actin and the CA interacts with, and activates Arp2/3 It is not yet clear whether the Wave/N-WASP VCA domain interacts with one of Arp2 or Arp3, or whether 2 VCA domains are required in a sequential activation process. Some data which have been used to determine these stoichemical ratios, using tagged and endogenous VCA proteins, which may affect their interaction with the ARP2/3 Complex proteins. Cross linking studies show that WAVE-1 and N-WASP have different complex formation effects, as WAVE-1 only brings the p14 subunit into close proximity of Arp2/3, whereas N-WASP brings both P14 and P19 subunits into close enough proximity to ARP2/3 to enable cross-linking. This suggests that WAVE-1 and N-WASP have differing conformational effects on ARP2/3, which may be associated with their different rates of nucleation. Together however, it would suggest that WAVE-1 may reduce actin nucleation rates compared to N-WASP. N-WASP has a high nucleating activity, whereas WAVE-1 had substantially lower rates of actin nucleation [13]. A reduction in actin polymerization would be a potential energy saving device, in that it would reduce the cells demand for ATP. Actin ATP is required for polymerization; hence a reduction in actin polymerization rates may reduce cell ATP requirements. A reduction in ARP2/3 complex activity when activated by WAVE is also consistent with MAPK phosphorylation of WAVE reducing actin polymerization [14]. This would suggest that there is a switch in nucleation/filamentation rates following preconditioning ischemia, which would be consistent with our previous observations [4].

To determine if ARP2/3 plays a role in ischemic conditioning in cortical cultures, ARP2/3 was inhibited for using viral transduction of CA peptides. Inhibition of ARP2/3 did not significantly alter cell death rates following PC or harmful ischemia, but completely blocked the protective effects of preconditioning compared to untreated cells (**Figure 4**). These findings indicate that the ARP2/3 complex may play a critical role in determining the protective effects of ischemic preconditioning. Throughout we have considered the biological effects of WAVE-1 and the ARP2/3 complex with respect to actin and dendritic spine formation. However there are additional cellular roles of actin and the ARP2/3 complex. Actin polymerization has also been shown to occur following DNA damage, and is essential for transcriptional responses to DNA damage. Rapid ischemic tolerance is not mediated by new protein synthesis events, but the role of transcription is less clear. It should be noted that ARP2 and ARP3 were observed localized to the nucleus. Additional roles for ARP2/3 have recently come to light, especially the role of ARP2/3 in regulation of intracellular transport of endosomes, and endo and exocytosis (WASH). Actin polymerization is believed to be necessary to generate sufficient force for vesicle endocytosis scission.

Actin branching is essential in the formation of the dendritic spine heads, and blocking Arp2/3 results in long thin dendrites/neuropodia [25]. Interestingly, long thin dendritic spines are observed in fragile-X syndrome, whereby loss of fragile-X mental retardation protein (FMRP) results in enhanced spine density in the hippocampus and cortex and an increase in seizures, similar to the human disease [26, 27]. Therefore these morphological characteristics of blocking Arp2/3 may determine the ability of the peptide inhibitors to block rapid ischemic tolerance.

WAVE-1 and ARP2/3 complex are essential components of cells, as observed by the embryonic and post natal lethality of such knockouts. Interestingly newer approaches have shown that ARP2/3 knockdown can activate senescence, although the VCA transductions did not significantly increase basal or ischemia induced cell death when we treated neurons (**Figure 4**). In a recent study WAVE-1 knockdown reduced dendritic spine density, and the resulting spines appear simple and non-bulbous. ARP2/3 inhibition results in elongated spines and a reduction in spine number [25]. Hence, there is consistency that inhibiting WAVE-1 and ARP2/3 affects spine morphology. What effect this has on NMDA receptor responses is not yet clear. Some small molecular inhibitors of ARP2/3 are now available including CK667, although the specificity of these compounds requires further validation: such compounds could be investigated in future studies.

This study identifies a role for actin regulating complex ARP2/3 and WAVE-1 in regulating rapid ischemic tolerance. What this study shows is that multiple and complex signaling cascades are activated following precondition-

ing ischemia. In addition, it suggests that selective sparing of proteins from ubiquitination can also influence rapid ischemic tolerance. However, further studies are warranted to further dissect the kinase pathways regulating Arp2/3 and to determine whether the effect of WAVE-1 and ARP2/3 is via actin in the dendritic spine or other cellular processes.

Acknowledgements

This work was supported by NIH grants NS50669 & NS59588 (Meller). Institutional support at MSM was provided by NIH/NCRR/RCMI Grant G12-RR03034 and U54 NS06-0659. We wish to acknowledge the kind gift of Arp inhibitory peptides from Dr. L Lanier, University of Minnesota, USA.

Disclosure of conflict of interest

None.

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