Interleukin-15 modulates the response of cortical neurons to ischemia

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ABSTRACT

Objective: Stroke is a major cause of death and disability in the United States. Current acute stroke therapy consists of clot-dissolving drugs, catheter-based interventions and physical rehabilitation. To date, there are no therapies that directly enhance neuronal survival after a stroke. Previous work from our lab demonstrated that Interleukin-15 (IL-15) peptide could rescue cardiomyocytes subjected to hypoxia. We sought to extend these findings to cortical neurons since IL-15 has been implicated to have an important role in neuronal homeostasis.

Methods: We have evaluated the effect of IL-15 peptide on primary cortical neurons derived from embryonic rats in vitro under conditions of anoxia and glucose deprivation, and in vivo following middle cerebral artery occlusion.

Results: IL-15 administration rescued neuronal cells subjected to anoxia coupled with glucose deprivation (AGD), as well as with reoxygenation. A hallmark of stroke is the ischemic microenvironment and associated oxidative stress, which results in DNA damage and ER stress, both of which contribute to neuronal cell damage and death. The expression of anoxia, ER stress, and DNA damage factors/markers was evaluated via western blot and correlated with the cellular survival effects of IL-15 in vitro. In addition, IL-15 effect of alleviating ER stress and increasing cell survival was also observed in vivo.

Interpretation: Our data indicate, for the first time, that administration of the pleiotropic factor IL-15 reduces neuronal cell death during AGD, which correlates with modulation of multiple cellular stress pathways.

1. Introduction

Stroke is a major cause of death and disability in the United States (Writing Group M et al., 2016). Current acute stroke therapy consists of clot-dissolving drugs and catheter-based interventions. Despite greater insights in the field of stroke research, medical treatment remains limited as there are no neuroprotective therapeutics available following an ischemic stroke (Nelson, 2007).

A hallmark feature of stroke is the ischemic microenvironment where cells experience decreased levels of oxygen ranging from hypoxia to anoxia, and glucose deprivation (Meloni et al., 2011), resulting in oxidative stress, DNA damage and endoplasmic reticulum (ER) stress (Li et al., 2011). To adapt and survive, cells respond to DNA damage and ER stress by inducing DNA damage response (DDR) pathways (Li et al., 2011) and the unfolded protein response (UPR) (Kim et al., 2008) respectively. However, prolonged DNA damage stress and/or ER stress may result in cell death (Kimura-Ohba and Yang, 2016).

ER stress has been demonstrated to play a major role in ischemic conditions (Xin et al., 2014). Previous studies have shown that brain ischemia results in hypoxia and hypoglycemia, which can result in ER stress that can lead to neuronal cell death (Tajiri et al., 2004). Ischemia causes oxygen deprivation and upregulation of the major hypoxia factor hypoxia-inducible factor 1-alpha (HIF1α) protein, which regulates hypoxic gene expression (Semenza et al., 1996). Ischemia also induces ER stress that initiates the UPR, activating pathways multiple pathways (Urra et al., 2016) that result in cell adaptation and survival at first. However, after prolonged ER stress, the UPR can become overwhelmed leading to cellular switch towards apoptosis (Reimertz et al., 2003).

In addition to ER stress, ischemic injury also leads to DNA damage, inducing both single strand breaks (SSB) and double strand breaks (DSB) (Li et al., 2011; Kimura-Ohba and Yang, 2016). Cells then respond by inducing the DDR pathway to attempt to correct any damage and...
promote survival. However, with prolonged and/or severe DNA damage, cells switch from survival to death pathways (Hong et al., 2004). Consequently, several research venues have addressed modulating DDR mechanisms to prevent cell death following ischemic insults (Kimura-Ohba and Yang, 2016). Interleukin-15 (IL-15) is a four α-helix bundle cytokine whose signaling effects are mediated through IL-15Rα, IL-2Rβ, and IL-2Rγ (Bullone-Paus et al., 2006). It has been shown to have pro-angiogenic roles in vivo (Angiulli et al., 1997) as well as anti-apoptotic roles in several different cell types, including lymphoid (Ma et al., 2006) and kidney epithelial cells (Shinozaki et al., 2002). Previous work from our lab has demonstrated that IL-15 peptide could rescue cardiomyocytes (CMs) subjected to hypoxia (Yeghiazarians et al., 2014) in vitro, and diminish scar size and improve heart function in a murine model of infarction in vivo, attributed to decreased cell death and increased vascularity (Ameri et al., 2020). Despite significant existing literature supporting the homeostatic and pro-survival effects of the IL-15 signaling pathway, its role as a neuroprotective agent in the setting of ischemia-related injury has not been investigated. Therefore, we sought to extend our previous findings and apply them to primary neurons in vitro subjected to stroke-like ischemic insults. This study is the first to demonstrate that administration of human IL-15 peptide modulates oxidative stress, DNA damage, and ER stress to increase survival of primary cortical neurons in ischemia.

2. Methods

2.1. Primary cortical neurons

All animal work was done in accordance with UCSF’s Institute on Animal Care and Use Committee (IACUC) policy. Cortical neurons were isolated from rat embryos on gestational day 17 as previously described (Nguyen and McQuillen, 2010). Briefly, pregnant Sprague Dawley rats (Charles River Laboratories, MA) were anesthetized and sacrificed. Embryos were removed and brain cortices were dissected in Hank’s solution (Thermo). Neurons were dissociated with MACS Neural Tissue Dissociation Kit (Miltenyi Biotec). Cells were plated at 50 × 10^3 cells per well on 96-well plates (Corning), or 2 × 10^6 cells on 6-well plates. Neurons were cultured in Neurobasal media (Thermo) supplemented with GlutaMAX (Invitrogen), Penicillin/Streptomycin (Invitrogen), B27 (Thermo), BDNF (PeproTech), and dbcAMP (Sigma-Aldrich). For glucose-free conditions, Neurobasal Media without Glucose was used. Ara-C (1 μM, Sigma-Aldrich) was used from 1 day-in-vitro (DIV) to DIV4 to produce <95% pure neuronal cultures.

2.2. Recombinant IL-15

Recombinant human IL-15 was purchased from Peprotech (Cat. 200-15, Peprotech, NJ, USA), reconstituted in deionized water at a stock concentration of 100 μg/mL and stored in −80 °C. For in vitro administration, IL-15 was diluted in Neurobasal media to reach working concentration. For in vivo administration, IL-15 was diluted in 0.85% saline solution.

2.3. Anoxia injury

To simulate anoxic environment, DIV7 neurons were incubated in an anaerobic jar (Oxoid, ThermoFisher). Air within the jar was flushed with 5% CO2, 5% H2, and 90% N2 gas (Airgas). A palladium catalyst (ThermoFisher) was placed within the chamber to scavenge residual oxygen, which was monitored by an oxygen sensor (Drager-Pac 3500) to maintain anoxia. To simulate ischemic conditions, glucose-free Neurobasal media was used, and remaining trace sources of glucose came from B27 supplement (~80 μM stock).

2.4. Oxidative stress

DIV7 neurons were incubated in normoxia or anoxia with the CellROX™ kit (Invitrogen), and assayed for ROS per manufacturer’s instructions. For direct ROS production, cells were incubated with media containing hydrogen peroxide (H2O2, Sigma-Aldrich) at an optimal concentration of 0.0001%.

2.5. Neuronal survival quantification

Neuronal survival was assessed using fluorescence-based LIVE/DEAD™ assay (Thermo). Calcein-AM and ethidium homodimer were used to label live and dead cells, respectively. Differential labeling of live versus dead cells allowed for discrimination under fluorescent microscopy. Each well was imaged using a Leica DMI 4000B inverted fluorescent microscope. The ratio of live-to-dead cells was quantified using IMARIS (Bitplane) software. Percent survival was determined relative to cells incubated under normoxic condition.

2.6. Immunoblot

Proteins were collected from cells lysed in RIPA buffer followed by sonication and centrifugation, and stored at −80 °C. Protein concentration was assessed using the Bradford assay (BioRad). 20 μg of proteins was separated on a 4–15% gradient gel (BioRad) via SDS-PAGE. Protein samples were run in triplicates from three distinct experiments. Proteins were transferred onto PVDF membrane (Millipore) and blocked with Odyssey Blocking Buffer-PBS (LI-COR) for 1 h at room temperature (RT). Membranes were incubated with the following primary antibodies overnight at 4 °C: IL-15Rα (Rb, Santa Cruz), IL-2Rβ (Rb, Sigma), HIF1α (Ms, Novus), MAP-LC3 (Ms, Santa Cruz), BRC1 (Rb, Abcam), γH2AX (Rb, Abcam), eIF2α (Ms, Cell Signaling), phosphorylated eIF2α (Rb, Cell Signaling), ATF4 (Rb, Proteintech), XBP1 (Rb, Abcam), phosphorylated JAK3 (Rb, Thermo), and Actin (Ms, Sigma). Membranes were then washed and probed with IRDye8® secondary antibodies (LI-COR) for 1 h at RT. Blots were washed and imaged using the Odyssey-Cx imaging system (LI-COR). Signal intensity was quantified using ImageJ software and normalized to control conditions.

2.7. Immunocytochemistry

Neurons were fixed with methanol at −20 °C then blocked with 5% BSA and 0.5% Tween in PBS for 1 h at RT. Cells were incubated with primary antibody overnight at 4 °C, which include: IL-15Rα (Rb, Santa Cruz), IL-2Rβ (Rb, Santa Cruz), IL-2Rγ (Rb, Santa Cruz), NeuN (Ms, EMD Millipore), cleaved caspase-3 (Rb, Cell Signaling), γH2AX (Rb, Abcam), and phosphorylated eIF2α (Rb, Cell Signaling). Cells were then exposed to secondary antibodies (Alexa Fluor, Thermo) for 1 h at RT, then were placed in Dapi-containing mounting media (Fluoromount-G, SouthernBiotech) for signal preservation. Images were taken with a Zeiss Axioimager.Z2 microscope equipped with a motorized stage.

2.8. Rat in vivo middle cerebral artery occlusion

All animal research was approved by UCSF IACUC. Middle cerebral artery occlusion (MCAO) was performed on Sprague Dawley rats at age P10 as previously described (Larpinthesaarp and Gonzalez, 2017). Briefly, rats were anesthetized with isoflurane (2% at 1.5 L/min) during the procedure. The MCA was occluded with a silicone-coated 6-0 nylon filament from Doccol Corporation (Sharon), and rats were allowed to recover. The filament was removed after 3 h for reperfusion. Rats were given a dose of recombinant human IL-15 (400 μg/kg body weight) intraperitoneally starting at time of reperfusion and every 4 h, up to 12 h after reperfusion. The timeline and dosage regimen were chosen based on IL-15 serum concentration as previously described (Ameri et al., 2020). Rat brains were harvested at P24. Brains were washed with PBS.
and fixed in 4% paraformaldehyde for 2 h. Samples were then placed in 30% sucrose solution for at least 12 h in −20 °C for cryoprotection. Brains were coronally sectioned at 50 μm thickness using a cryostat (Leica). Tissues were mounted onto Superfrost slides and stored at −80 °C. A total of 4 rats receiving MCAO, 4 rats receiving MCAO and IL-15, and 4 rats receiving sham operations were used in this study. No mortality was observed.

2.9. Immunohistochemistry and microscopy

Immunohistochemistry was performed as previously described (Nguyen et al., 2017). Briefly, mounted brain slides were washed in PBS. Slides were rehydrated through an ethanol gradient and stained with cresyl violet. For immunostaining, citrate antigen retrieval was performed (pH 6.0) at 95 °C for 5 min and allowed to cool to RT. Tissues were then incubated in blocking buffer (10% goat serum, 0.1% Triton X-100 in PBS) for 1 h followed by primary antibody incubation overnight. The following primary antibodies were used: IL-15Rα, IL-2Rβ (Rb, Santa Cruz), NeuN (Ms, Abcam), or phosphorylated eIF2α (Rb, Cell Signaling). Tissues were stained with secondary antibodies (Alexa Fluor, Thermo) for 1 h, then placed in Dapi-containing mounting media (Fluoromount-G, Southern Biotech). Images were taken with a Zeiss Axioimager.Z2 microscope.

2.10. Statistical analysis

Investigators performing tests and analysis were blinded to treatment group. Data are calculated as means ± SEM. Mean values were compared using Student’s t-test as well as one-way ANOVA, for pairs and live/dead quantification with multiple timepoints, respectively. p < 0.05 was considered statistically significant.

3. Results

3.1. IL-15 receptor subunits are expressed in cortical neurons

IL-15 is known to mediate its effect through binding to IL-15Rα, IL-2Rβ, and IL-2Rγ (Bulfone-Paus et al., 2006). To investigate the expression of IL-15 receptor subunits, immunocytochemistry analysis was performed on rat cortical neurons in vitro (Fig. 1A). Immunopositive staining was co-localized to the neuronal marker NeuN-positive cells, and immunoblots revealed the presence of IL-15Rα and IL-2Rβ. In addition, brain tissue from P17 rats that were exposed to MCAO was stained for IL-15Rα or IL-2Rβ, and NeuN (Fig. 1B). The cortical region revealed co-localization of both subunits to NeuN in control and MCAO brains, with more diffuse staining in injured brains.

3.2. IL-15 protects against neuronal cell death in stroke-like conditions in vitro

To investigate the effect of IL-15 on cortical neurons under anoxia coupled with glucose deprivation (AGD) conditions that mimic stroke, cultured neurons received glucose-free media with any remaining glucose (2 μM) coming from supplements, and transferred to a vacuum-sealed anaerobic jar. After 24 h of incubation in AGD, fewer than 10% neurons survived (Fig. 2 A), compared to 50% survival under normal culture conditions, as previously reported (Nguyen and McQuillen, 2010). AGD incubation confirmed increased protein levels of the hypoxia marker HIF1α, ER-stress factor XBP1, DNA damage factors γH2AX and Ub-γH2AX, as well as autophagy marker MAP LC3 type-II (Fig. 2 B).

We next looked at whether IL-15 could be neuroprotective. We first determined the optimal dose of IL-15 that gave the greatest difference in cell survival to be 100 ng/mL. We found that administration of IL-15 peptide resulted in significantly increased cell survival starting at 12 h.
reduce neuronal death by adding IL-15 peptide at 4, 6, or 8 h after cells oxygenation was significantly increased with IL-15 (25% vs physiologic levels of glucose (5 mM). Neuronal survival after 16 h of re-oxygenation, mimicking reperfusion following catheter-based interventions. Neurons were incubated for 8 h in AGD then subsequently re-oxygenated by placing them in a standard incubator and adding physiologic levels of glucose (5 mM). Neuronal survival after 16 h of re-oxygenation was significantly increased with IL-15 (25% vs 17%, p < 0.001; Fig. 2E). This demonstrates for the first time that IL-15 can attenuate neuronal death in different stroke-like settings in vitro.

3.3. IL-15 is neuroprotective by diminishing apoptosis and alleviating stress

To determine potential modes of neuroprotection, we first examined cell death in cortical neurons incubated in AGD versus cultures that were administered IL-15. Live/dead cell staining indicated increased live (Calcein-AM, green) and diminished dead cells (Ethidium homodimer, red, Fig. 3A; quantification, Fig. 2C). In addition, we also immunostained neurons for cleaved caspase-3 (C. Casp3), a marker of apoptosis (Fig. 3B). Cultures in AGD treated with IL-15 showed fewer neurons positive for C. Casp3 compared to nontreated (64.4% vs 45.8%, p < 0.02), suggesting decreased apoptosis.

We next looked at BRCA1 expression in cortical neurons. BRCA1 facilitates DNA repair processes as well as cell cycle checkpoint arrest and apoptosis (Li et al., 2011). Under specific stresses, caspase 3-mediated cleavage of BRCA1 results in lower molecular weight BRCA1-p90 protein, which can act as a mediator of apoptosis (Dizin et al., 2008). Indeed, we detected a 90 kDa cleaved form of BRCA1 under AGD conditions, which can act as a mediator of apoptosis (Fig. 3C). As we found BRCA1-207 kDa disappearance in AGD, we sought to further explore the role of DNA damage under such conditions. Previous work has established that ischemia can cause DNA damage (Chen et al., 1997), and if left unalleviated, cells will die (Kimura-Ohba and Yang, 2016). IL-15 has previously been described to modulate DNA damage (Gupta et al., 2019). To determine if IL-15 also affected DNA damage under AGD, we investigated modified forms of the histone H2AX, including phosphorylation at Ser139 (referred to as γH2AX) to epigenetically mark DNA lesions for DDR (Bergink and Jentsch, 2009).

Our results showed that under normoxia, neurons expressed low levels of γH2AX and Ub-γH2AX, but AGD
Fig. 3. IL-15 decreases neuronal apoptosis in vitro.
A) Representative images showing live (green, Calcein AM) and dead (red, Ethidium homodimer) cells incubated for 16 h in different conditions. Note increased live and decreased dead cells with IL-15. Scale bar, 200 μm.
B) Immunostaining of cleaved caspase 3 (C.Casp3, red) incubated in AGD with (top) or without (bottom) IL-15 shows a decrease in cells positive for C.Casp3 with IL-15. Scale bar, 200 μm.
C) Immunoblots of BRCA1 in cortical neurons. The full length (207 kDa) form is only detected in Nx conditions, while the cleaved (90 kDa) form appears in AGD conditions and correlates with increased apoptosis; IL-15 decreases cleaved BRCA1. Dotted lines denote splicing from membrane scan.
D) Immunoblots of the DNA damage markers γH2AX (16 kDa) and Ub-γH2AX (30 kDa) in cortical neurons. The increase in expression corresponds to AGD incubation, and IL-15 administration lowers expression. N = 3 per antibody. Dotted lines denote splicing from membrane scan.
incubation resulted in both being upregulated (Fig. 3D). Finally, IL-15 administration reduced levels of both (Fig. 3D). In addition to immunoblot analysis, immunocytochemistry also indicated that γH2AX was detectable in neurons during AGD, and that IL-15 reduced this expression (data not shown). Taken together, these data show that IL-15 can modulate DNA damage stress in neurons under AGD conditions.

Another important factor contributing to DNA damage is oxidative stress, which can lead to both necrosis and apoptosis in ischemic tissues (Manzanero et al., 2013). Using an ROS assay, we found that incubation in AGD upregulated ROS production when compared to normoxia, and that IL-15 administration could significantly decrease ROS (AGD, 2.15 ± 0.31 vs AGD + IL-15, 0.91 ± 0.11, p < 0.05, Fig. 4A). This led us to ask whether IL-15 directly reduced oxidative stress or decreased ROS production was a secondary effect. Therefore, we incubated neurons in media with H2O2 to generate free radicals, and quantified the percentage of live cells after 90 min. We found that this concentration was sufficient to cause cell death, and more importantly that IL-15 administration could enhance cell survival (71 ± 5.6% vs 97 ± 7.3%, p < 0.05, Fig. 4B). Additionally, ATF4 has been shown to be an oxidative stress-inducible, pro-death factor in neurons (Lange et al., 2008). Indeed, in our in vitro system we confirmed that H2O2 greatly increased ATF4 expression (Liu, 2020), and that IL-15 administration significantly

![Fig. 4. IL-15 prevents ROS and ER stress-mediated injury.](image)

A) Quantification of ROS assay showing oxidative stress after 2 h incubation in AGD. IL-15 significantly decreases intensity. N = 4.

B) Viability quantification after 90 min incubation in H2O2 shows a significant decrease in live neurons, and is rescued with IL-15 administration. N = 4.

C) Quantification and immunoblots of ATF4 protein expression (38 kDa) following 90 min incubation in H2O2 shows significant increase over normoxia, and IL-15 administration significantly lowered expression. N = 3.

D) Immunoblots of ER stress markers phosphorylated eIF2α (P-eIF2α, 40 kDa), ATF4 (38 kDa), and XBP-1 (56 kDa) in cortical neurons. All were increased in AGD versus Nx, and partially decreased in AGD + IL15 versus AGD. N = 3 per antibody. Dotted lines denote splicing from membrane scan. *, p < 0.05; **, p < 0.005.
lowered it (Fig. 4C).
Finally, as ATF4 is implicated in the PERK/ATF4/CHOP ER stress pathway (Urra et al., 2016; Bindra et al., 2005), we sought to explore whether ER stress was impacted by IL-15. Previous studies have shown IL-15 can decrease ER stress in skeletal muscles (Yang et al., 2015), while neuronal homeostasis is affected by ER stress in ischemic conditions (Taylor, 2016). During ER stress, eIF2α becomes phosphorylated (P-eIF2α), resulting in translational blockade; the UPR will subsequently attempt to correct the rate of protein translation to retain protein homeostasis or proteostasis, and if achieved eIF2α dephosphorylation occurs to resume translation (Han et al., 2013). Other factors become induced, including XBP1, and their roles depend on proteostasis conditions (Han et al., 2013). We evaluated via immunoblot analysis the expression of the ER stress-related factors P-eIF2α, ATF4, and XBP1 at 16 h (Fig. 4D), and found that IL-15 reduced expression levels of all three factors. Taken together, the data show that IL-15 can modulate ER stress and promote cell survival.

3.4. IL-15 neuroprotection acts through JAK3 signaling

Previous work has shown that IL-15-mediated cardiomyocyte survival after hypoxia was attributed to the JAK3/STAT3 pathway (Yeghiazarians et al., 2014). Furthermore, ischemic stroke could result in increased levels of JAK3 expression in neurons (DeMars et al., 2017). To determine whether the neuroprotective effects of IL-15 involve JAK3 activity, we used JAK-specific pharmacological inhibitors: AZD1480, a JAK2-specific inhibitor, and PF-Malonate, a JAK3-specific inhibitor. In AGD, IL-15 caused phosphorylated JAK3 (pJAK3) expression to increase, which was abolished with PF-Malonate administration (Fig. 5A). Next, we investigated whether blocking pJAK3 signaling would affect IL-15-mediated ER-stress modulation, by proxy of P-eIF2α expression, and neuroprotection, via cell survival assays. IL-15 significantly decreased P-eIF2α (Fig. 5B, C) and cell death (Fig. 5D) in the presence of AZD1480, but not in the presence of PF-Malonate. These results support IL-15 can modulate ER stress and protect neurons through JAK3 signaling.

3.5. IL-15 preserves cortical neurons in rat brains subjected to MCAO

Finally, we sought to investigate whether the neuroprotective effects of IL-15 in vitro could be translated to an in vivo stroke model. We used the MCAO in P10 postnatal rats injury model as it is a typical animal stroke model that our lab has experience with previously (Larpthaveesarp and Gonzalez, 2017). IL-15 did not have gross deleterious effects (Fig. 6A) and increased NeuN-positive neuronal cells in the cortical ischemic areas (Fig. 6B). To verify whether ER stress was also affected by IL-15 in vivo, we stained for P-eIF2α and found that IL-15-treated brains had lower expression (Fig. 6C). These results indicate that IL-15 modulates ER-stress and diminished cortical neuronal loss following MCAO. Thus, we have shown that our in vitro findings on IL-15-mediated neuroprotection were in part translated to an in vivo model of stroke.

Fig. 5. IL-15 neuroprotection is dependent on phosphorylated-JAK3 signaling.
A) Representative immunoblots showing phosphorylated-JAK3 (pJAK3, 125 kDa) expression in cortical neurons after incubation in various conditions with or without PF-Malonate (PF-Mal.), a JAK3-specific inhibitor. Note that pJAK3 expression is upregulated by IL-15, but remains unchanged in the presence of PF-Malonate. N = 3 per antibody. Dotted lines denote splicing from membrane scan.
B) Representative immunoblots showing expression of pJAK3 (125 kDa) and P-eIF2α (40 kDa) in cortical neurons treated with different JAK inhibitors in AGD. AZD, AZD1480: a JAK2-specific inhibitor. pJAK3 was increased while P-eIF2α was decreased in AGD + IL-15 + AZD versus AGD + AZD conditions, but neither markers changed expression between AGD + IL-15 + PF-Mal. and AGD + PF-Mal.
C) P-eIF2α quantification reveals significant decrease in expression with the addition of IL-15 in neurons treated with AZD1480, but not with PF-Malonate. N = 3. D) Live/dead quantification of neurons following 16 h incubation in AGD. There was a significant increase in live cell percentage with AZD1480, but no difference with PF-Malonate. N = 3. *, p < 0.05, **, p < 0.005.
4. Discussion

IL-15 is a pleiotropic factor expressed in various tissues. Prior work from our lab demonstrated that exogenous IL-15 promotes cell survival in cardiomyocytes exposed to hypoxia (Yeghiazarians et al., 2014), and improves heart function in a mouse model of infarction (Ameri et al., 2020). We have extended these findings to show for the first time that IL-15 confers neuroprotective effects on cultured neurons exposed to AGD, mimicking stroke.

In vitro IL-15 administration improved cortical neuron survival, which correlated to alleviation of ER stress. This in vitro effect was also observed in vivo. Cells under ischemic stress experience oxygen deprivation and nutrient deficiency, leading to ER stress. The role of ER stress response in ischemic neurons has been extensively studied, both to initially promote cell survival (Halterman et al., 2010) or cause apoptosis under prolonged stress (Xu et al., 2005). Cells adapt by initiating the UPR and enabling survival (Halterman et al., 2010) through three main pathways involving IRE1-XBP1, PERK-P-eIF2α-ATF4, and ATF6 (Kim et al., 2008). However, too much ER stress can overwhelm their adaptive capacity (Galehdar et al., 2010) and lead to apoptosis, for example with XBP1 promoting neuronal cell death (Oiishi et al., 2015). ER stress modulation has been considered as therapeutic strategies in both cardiovascular disease and stroke (Xin et al., 2014). Indeed, IL-15 in AGD neuronal cultures reduced expressions of both XBP1 and P-eIF2α, which suggests alleviation of ER stress thereby delaying apoptosis, although we have yet to explore ATF6. As we observed increased expression of these markers after 16 h of incubation in AGD, they are likely involved in promoting cell death rather than survival at this timepoint. Interestingly, AGD can induce ROS (Sohn et al., 2017), which promotes ER stress. Since IL-15 diminished ROS, possibly alleviating re-oxygenation-mediated oxidative stress, this could also explain reduced ER stress in neurons.

In addition, DNA damage is another hallmark of ischemic stroke (Li et al., 2011). The response to excessive DNA damage involves endonuclease activation that leads to DNA strand breaks and fragmentation, followed by apoptosis (MacManus et al., 1999). ROS also causes oxidative DNA damage, occurring during early onset of ischemia or reperfusion (Chen et al., 1997). Indeed, we detected upregulation of γH2AX and Ub-γH2AX in AGD, and observed that IL-15 administration reduced expression of both. In addition, IL-15 also reduced BRCA1-p90 accumulation, a cleaved form of BRCA1 involved in mediating apoptosis (Dizin et al., 2008). This result correlated with our observation that IL-15-treated neurons expressed less cleaved caspase 3, which supports its anti-apoptotic role. Interestingly, BRCA1-207 kDa disappeared shortly after AGD incubation, which IL-15 failed to prevent. Previous reports have noted BRCA1 could rescue neurons via antioxidant response pathways after ischemia/reperfusion injury models (Xu et al., 2018). IL-15 may therefore act on different pathways to reduce ROS, and further work is needed to explore potential additive or synergistic roles of BRCA1 with IL-15 administration. Taken together, our results suggest that IL-15 acts on multiple pathways involved in ischemic injury, further validating its pleiotropic effects.

In our study we saw that AGD downregulated full-length BRCA1 expression, and IL-15 had no effect on it. As previously reported, in cancer cells hypoxia can downregulate BRCA1, for example through

Fig. 6. IL-15 confers neuroprotection and decreased ER stress in rat MCAO brains. A) Representative images of cresyl violet stain of coronal sections (top) and gross anatomical brains (bottom) from MCAO rats at P24. Left, ipsilateral (injured) hemisphere, right, contralateral (uninjured) hemisphere. Saline was used as vehicle. White box indicates representative cortical regions analyzed in subsequent images. Note increased ipsilateral hemisphere area in IL15-treated brains compared to vehicle. B) Immunostains of NeuN and Dapi in the ischemic region of MCAO brains show increased cells positive for NeuN in IL15-treated sections compared to vehicle. Images are representative of 3 biological replicates. Scale bar, 150 μm. C) Immunostains of P-eIF2α in the ischemic region of MCAO brains show a decrease in immunopositive cells in brains treated with IL-15 compared to brains treated with vehicle. Scale bar, 50 μm.
transcriptional repression of E2F pathways and promoter occupancy redistribution (Bindra et al., 2005; Seifeddine et al., 2008). In addition to cancer cells, hypoxia has also been reported to downregulate BRCA1 in hepatic stellate cells, although the consequences remain unclear (Shi et al., 2007). Mitochondrial dysfunction is a hallmark of ischemia/reperfusion injury (Bakhavachalam and Shannagum, 2017), and was recently shown to cause proteasomal degradation of BRCA1 in breast cancer cells, potentially resulting in increased DNA damage following loss of BRCA1 (Miyahara et al., 2021). Ischemia can also affect proteasomal activity in neurons leading to degradation of proteins such as PCK-\(\gamma\) (Matsumoto et al., 2004) and AKAP121 (Carlucci et al., 2008). Collectively, these papers indicate a link between ischemia, mitochondrial dysfunction, and proteasomal degradation of BRCA1. Whether ischemic injury in cortical neurons may cause proteasome-mediated degradation of BRCA1 contributing to cell survival versus increased mutation and cell death is subject to future investigation.

We have shown that administration of IL-15 can help reduce cortical neuronal death in vitro by alleviating oxidative, DNA and ER stress, and diminishing apoptosis. This mechanism depends in part on JAK3 signaling, as reported by previous studies (Krolop et al., 2016). Interestingly, when we applied either JAK2 or JAK3 pharmacological inhibitors, we observed a slightly lower neuronal survival ratio at 16 h compared to our previous results without inhibitors. This may be attributed to potential JAK2/3 roles in neuroprotection (Chiba et al., 2009), and additional experiments such as knockdown models would be required to confirm the exact role of JAK3 in the pro-survival effect of IL-15.

Our data indicate that the protective effects of IL-15 in neurons remain with delayed administration from onset of AGD, and after reoxygenation. These results are encouraging from a translational clinical standpoint. Stroke patients present for medical care hours after their initial symptoms, and there exist limited therapies. Even for patients treated with reperfusion therapies, there are currently no medical therapies available for neuroprotection. IL-15 might be of potential benefit in both subsets of patients. Importantly, the effects of IL-15 on alleviating ER stress and increasing cell survival in vitro was also observed in vivo, where IL-15 administration in MCAO rats decreased expression of the ER-stress marker P-eIF2\(\alpha\) and diminished neuronal loss in the penumbra of injury in the cortex. To examine any therapeutic potential for IL-15 in rodent MCAO models, we are currently planning larger in vivo studies with increased sample size to address issues such as: detailed stereologic investigation to determine volumetric analysis of whole brain and specific brain regions, as well as functional outcomes linked to behavioral changes such as motor coordination and learning and memory tests.

Previous studies have reported an injurious role of IL-15 signaling in transgenic mice overexpressing IL-15 that received ischemic stroke, involving increased glial cell activation and secretion of IL-15 resulting in greater infiltration of CD8+ T and NK cells to the site of injury (Li et al., 2017). In addition, other studies have shown that an IL-15KO transgenic mouse model showed diminished infarct size via decreased CD4+ T, CD8+ T and NK cells in the brain (Lee et al., 2018). The primary role of IL-15 is well-documented as an inflammatory cytokine, and therefore agrees with these results. Indeed, transgenic mice lacking IL-15R\(\alpha\) displayed increased harmful astroglisis and microglisis when challenged with LPS (Wu et al., 2010). While our data seem in conflict at first glance, such is not the case as we have focused on IL-15 effect on neuronal survival. We propose that our results differ from previous studies due to our use of IL-15 in an acute manner, as opposed to the global or chronic effect seen in transgenic animals. In addition, IL-15 has a short half-life as seen in our studies (data not shown), potentially elucidating a lack of acute immune response in our in vivo results. Finally, our study is the first to show a direct effect of IL-15 signaling on cortical neurons, as opposed to an indirect effect on brain tissues via inflammation. Further work will address to what extend acute IL-15 administration affects overall immune responses following ischemic stroke.

In addition to glossis and inflammation, IL-15 may also affect glucose metabolism. For example, IL-15 modulated glucose metabolism in skeletal muscle cells (Nadeau et al., 2019), cardiomyocytes (Hennigan et al., 2019), and CAR-T cells (Alizadeh et al., 2019). It is therefore likely that IL-15 can potentially modulate metabolic pathways during AGD. We are currently pursuing further animal studies to address these issues, as well as the pharmacokinetics of therapy and behavioral improvements after stroke to evaluate potential benefits of IL-15 administration.

CRediT authorship contribution statement

VN, KH, MF, RG, AL performed experimental procedures. VN, KA, YY conceived the project, designed research, and planned all experiments. VN, KA, KH, FG analyzed data and prepared figs. VN, KA, KH, FG, YY wrote the article. All authors read and approved the final version.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Yeghiazarians holds patent 9084761: “Use of Interleukin-15 to treat cardiovascular diseases.”

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