

Induction of IRG1 following ischemic stroke promotes HO-1 and BDNF expression to alleviate neuroinflammation and restrain ischemic brain injury

Ping-Chang Kuo Indiana University School of Medicine
Wen-Tsan Weng Indiana University School of Medicine
Barbara A. Scofield Indiana University School of Medicine
Destin Furnas Indiana University School of Medicine
Hallel C. Paraiso Indiana University School of Medicine
Holiana University School of Medicine
Indiana University School of Medicine
Jui-Hung Yen (≤ jimyen@iu.edu) Indiana University School of Medicine

Research

Keywords: IRG1, ischemic stroke, neuroinflammation, microglia, HO-1, BDNF, D3T

Posted Date: May 26th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-27606/v1

License: (a) This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Abstract Background

Inflammatory stimuli induce immunoresponsive gene 1 (IRG1) expression that in turn catalyzes the production of itaconate through diverting cis-aconitate away from the tricarboxylic acid cycle. The immunoregulatory effect of IRG1/itaconate axis has recently documented in LPS-activated mouse and human macrophages. In addition, dimethyl itaconate, an itaconate derivative, was reported to ameliorate disease severity in the animal models of psoriasis and multiple sclerosis. Currently, whether IRG1/itaconate axis exerts an immunomodulatory effect in ischemic stroke is unknown. Thus, we investigated whether IRG1 plays a role in modulating brain injury in ischemic stroke. In addition, the molecular mechanisms underlying the protective effects of IRG1 in ischemic stroke were elucidated.

Methods

Wild type (WT) C57BL/6 and *IRG1^{-/-}* mice were subjected to 40 minutes middle cerebral artery occlusion (MCAO). Ischemic brain injury, microglia (MG) activation and peripheral immune cell infiltration of the ischemic brain were assessed. Furthermore, the expression of HO-1 and BDNF in the ischemic brain was measured. Finally, *IRG1^{-/-}* MCAO mice were administered with D3T, an Nrf2/HO-1 pathway inducer, to determine its effects on cerebral BDNF expression and ischemic brain injury.

Results

We observed IRG1 was highly induced in the ischemic brain of WT but not *IRG1^{-/-}* MCAO mice. Strikingly, *IRG1^{-/-}* MCAO mice exhibited exacerbated brain injury with enlarged cerebral infarct, enhanced MG activation, and elevated immune cell infiltrates compared to WT MCAO controls. Further analysis of molecular mechanisms underlying the protective effects of IRG1 in ischemic stroke revealed that IRG1 promoted HO-1 and BDNF expression to restrain ischemic brain injury, as *IRG1^{-/-}* MCAO mice exhibited reduced HO-1 and BDNF expression in the ischemic brain compared to WT MCAO controls. Notably, D3T, an Nrf2/HO-1 pathway inducer, promoted BDNF expression and lessened ischemic brain injury in *IRG1^{-/-}* MCAO mice.

Conclusions

We demonstrate that the induction of IRG1 following ischemic stroke may serve as an endogenous protective mechanism to restrain ischemic brain injury, and that may be mediated through the protective effect of IRG1 on the induction of HO-1 and BDNF expression in the ischemic brain. Thus, our study suggests that targeting IRG1 may represent a novel approach for the treatment of ischemic stroke.

Introduction

Stroke is a leading cause of disability in the U.S. and worldwide. More than 80% of stroke cases belong to ischemic stroke, in which the occlusion of cerebral blood vessels initiates the acute phase of cerebral injury followed by excitotoxicity and oxidative damage (1, 2). Tissue plasminogen activator is an FDA-approved therapy for ischemic stroke, and it functions to dissolve the clots that allows reestablishment of cerebral blood flow. However, reperfusion induces reactive oxygen species (ROS) generation and recruits peripheral inflammatory immune cells into the ischemic brain, leading to enhanced neuroinflammation and ischemic brain injury exacerbation (3, 4).

Inflammatory stimuli induce immunoresponsive gene 1 (IRG1) expression (5–7). IRG1 has been shown to catalyze the production of itaconate through diverting cis-aconitate away from the tricarboxylic acid cycle (TCA) cycle (8). The immunoregulatory effect of itaconate was recently documented in macrophages (MΦ). For instance, the study showed that itaconate promoted Nrf2 activation, a key player in the antioxidant defense, in mouse and human MΦ stimulated with LPS (6, 7). In addition, *IRG1^{-/-}* MΦ activated with LPS exhibited augmented inflammatory responses compared to wild type MΦ stimulated with LPS (5). Furthermore, dimethyl itaconate (DMI), an itaconate derivative, was reported to inhibit IL-17-induced IkBς activation in keratinocytes and ameliorate disease severity in the imiquimod-induced psoriasis animal model (9). Moreover, DMI was shown to offer a protection against myocardial and cerebral ischemic/reperfusion injury in animal models (10, 11). Finally, DMI was recently reported by our group to suppress neuroinflammation and ameliorate disease severity in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (12).

Currently, whether IRG1 exerts a protective effect against ischemic stroke remains unknown. Thus, we accessed whether IRG1 is induced in the ischemic brain following middle cerebral artery occlusion (MCAO), and investigated whether IRG1 exerts an immunomodulatory effect in ischemic brain injury. We then deciphered the molecular mechanisms underlying the protective effects of IRG1 in ischemic stroke. In this study, we report for the first time that the induction of IRG1 following ischemic stroke serves as an endogenous protective mechanism to alleviate neuroinflammation and restrain ischemic brain injury, and that may be mediated through the protective effect of IRG1 on the induction of HO-1 and BDNF expression in the ischemic brain. Thus, our findings suggest that targeting IRG1 may represent a novel approach for the treatment of cerebral ischemia.

Material And Methods

Mice

IRG1 ^{-/-} and its corresponding wild type (WT) control C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal experimental procedures were approved by the Purdue Animal Care and Use Committee (PACUC) and performed in strict compliance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All mice were housed and bred in the animal

facility with controlled humidity, temperature, and 12h: 12 h light: dark cycle with free access to food and water.

Reagents

Triphenyltetrazolium chloride (TTC) and Lipopolysaccharide (LPS; *Escherichia coli* 055:B5 were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 anti-mouse CD45 (clone: 30-F11), APC anti-mouse CD11b (clone: M1/70), PE/Cy7 anti-mouse CD86 (clone: GL-1) antibodies for FACS analysis were purchased from BioLegend (San Diego, CA). Alexa Fluor 488 anti-mouse Iba1 (EPR16588) antibody for IHC and anti-mouse IRG1 (EPR22066) antibody for western blot analysis were purchased from Abcam (Cambridge, MA). Anti-mouse HO-1 (10701-1-AP) antibody for IHC and western blot analysis, and anti-mouse BDNF (28205-1-AP) antibody for western blot analysis were purchased from Proteintech (Chicago, IL). D3T was prepared as previously described (13).

Primary microglia (MG) and macrophage (M ϕ) culture

Primary MG were generated from P1-P2 neonatal mice previously described (14). Briefly, cerebral cortical cells from P1-P2 neonatal mice were dissociated and plated in 75-cm² culture flasks in DMEM/F12 supplemented with 10% FBS. The medium was removed and replenished with complete medium containing 10 ng/ml GM-CSF on day 4 and 8 after plating. MG were harvested by shaking the flasks at 250 rpm at 37 °C for 30 minutes on day 12. MΦ were generated from bone marrow cells as previous described (15). Briefly, bone marrow cells were cultured in RPMI 1640 medium containing 10 ng/ml M-CSF. Cells were replenished with fresh media containing 10 ng/ml M-CSF at day 3 and collected for experiments at day 7.

Middle cerebral artery occlusion (MCAO) model

Adult male (3–4 months old) C57BL/6 and *IRG1^{-/-}* mice were used for cerebral ischemia experiments as previously described (16). The transient ischemic stroke was induced by an intraluminal suture occlusion model. Briefly, mouse was anesthetized by isoflurane with a mixture of 70% compressed air and 30% oxygen. During the surgical procedure, the body temperature of the animal was maintained at ~ 37 °C. Cerebral blood flow (CBF) was measured by laser Doppler flowmetry (Moor Instrument VMS-LDF2) to confirm the reduction of CBF following suture insertion. After 40 minutes occlusion, the intraluminal suture was removed to reestablish cerebral blow flow. Mice with a total reduction of CBF more than 80% were included in this study.

Infarct volume measurements

The infarct volume of the ischemic brain was determined by TTC staining. Briefly, the ischemic brain harvested from MCAO mice was subjected to 2-mm coronal slicing with a rodent brain matrix followed by 1% TTC staining. After staining, the brain sections were scanned and the infarct volume was calculated by ImageJ as previously described (16).

Isolation of mononuclear cells from mouse brain

Mice subjected to MCAO were anesthetized and perfused with PBS transcardially. After removing the meninges, olfactory bulb and cerebellum, the forebrains were homogenized with 1X HBSS buffer followed by filtration through a 70-µm nylon cell strainer. After centrifugation, cells were resuspended in 30% Percoll underlayering with 70% Percoll. Following centrifugation, the mononuclear cells were then isolated from the interface between 30% and 70% Percoll. The isolated cells were then stained with antibodies and analyzed by flow cytometer (BD FACSVerse).

Real-time RT-PCR

The expression of *Irg1, Ho-1, and Bdnf* was detected by quantitative PCR (Q-PCR) as previously describe (17). The primers used were *Irg1*: sense 5'-GCAACATGATGCTCAAGTCTG-3' and antisense 5'-TGCTCCTCCGAATGATACCA-3'; *Ho-1*: sense 5'-GCTGGTGATGGCTTCCTT GT-3' and antisense 5'-ACTGGGTTCTGCTTGTTGCG-3'; *Bdnf*: sense 5'-TGCAGGGGC ATA GACAAAAGG-3' and antisense 5'-CTTATGAATCGCCAGCCAATTCTC-3'.

Western Blot Analysis

Protein samples were prepared in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF and 1X protease inhibitor cocktail] with 0.3% SDS (brain tissue) or 0.1% SDS (cultured cell). The protein concentrations were detected by PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Protein electrophoresis was conducted on 10% or 12% SDS-PAGE. After transferred to polyvinylidene difluoride membranes (Millipore Temecula, CA), the blots were reacted with specific antibodies and detected by using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Temecula, CA).

Immunohistochemistry

Brain samples were dissected and fixed with 4% PFA in PBS at 4 °C overnight. After 6% and 30% sucrose dehydration, brain samples were embedded in OCT and cut into 40 µm cryosections. Cryosections were air-dried at room temperature (RT) and permeabilized in PBS containing 0.5% Triton X-100 for 30 minutes. Following blocking with goat serum (5% goat serum, 0.25% Triton X-100 in PBS) at RT for 1 hour, sections were incubated with primary HO-1 antibody at 4 °C overnight. After wash, sections were then stained with secondary antibody for 2 hours followed by Alexa Fluor 488 anti-Iba1 antibody staining for 2 hours at RT. After wash, samples were coverslipped with ProLong Gold antifade mountant containing DAPI. The Z-stack images of Iba1 and HO-1 co-localization were visualized by confocal microscopy (Fluoview FV10i, Olympus). The number of Iba1⁺ cells per mm² was counted and quantified by ImageJ.

Statistical analysis

All results are given as mean \pm SEM. The normal distribution of the data in each group was confirmed by Shapiro-Wilk normality test. Comparisons between two groups were done by unpaired *t* test, whereas comparisons between multiple groups were done by one-way ANOVA followed by Bonferroni *post hoc* test. Statistical significance was determined as *p* < 0.05.

Results

Ischemic stroke induces IRG1 expression in the ischemic brain, however IRG1 deficiency exacerbates brain injury following ischemic insults

To evaluate whether ischemic stroke induces IRG1 expression, C57BL/6 mice were subjected to MCAO, and the ischemic brains were harvested and subjected to Q-PCR analysis to assess IRG1 expression. Our results showed that ischemic stroke induced IRG1 expression in the ischemic brain in which IRG1 was highly upregulated in the ipsilateral but not contralateral hemisphere (Fig. 1A).

To further evaluate whether the induction of IRG1 following ischemic stroke plays a role in modulating ischemic brain injury, we thought to compare the level of ischemic brain injury between $IRG1^{-/-}$ mice and its corresponding WT controls. To do that, we first determined whether IRG1 deficiency may result in alteration of the cerebrovasculature in IRG1^{-/-} mice, as it would affect the outcome of ischemic brain injury following ischemic insults. Thus, the level of CBF in naïve WT and IRG1^{-/-} mice was measured and compared. Our results showed no significant differences in the reading of CBF between WT and IRG1^{-/-} mice (Fig. S1). In addition, the blood-brain barrier (BBB) integrity of *IRG1^{-/-}* mice was assessed and compared with that of WT controls. We observed WT and $IRG1^{-/-}$ mice exhibited a similar level of BBB integrity, as there was no increased Evans blue leakage detected in the brain of IRG1^{-/-} mice compared to that of WT controls (Fig. S2). Collectively, our results demonstrate that the CBF and BBB integrity are comparable between WT and IRG1^{-/-} mice, suggesting that IRG1 deficiency does not alter the cerebrovasculature in *IRG1^{-/-}* mice. With that, we then assessed and compared the severity of ischemic brain injury between WT and IRG1^{-/-} MCAO mice. Although the physiological parameters of male WT and $IRG1^{-/-}$ mice during MCAO were comparable (Table S1), WT and $IRG1^{-/-}$ MCAO mice displayed a different pattern of brain injury following ischemic stroke. In WT MCAO mice, the infarct was mainly observed in the striatum of ischemic brain, however in IRG1^{-/-} MCAO mice, the infarct was further extended to the cortex of ischemic brain (Fig. 1B top). Further analysis of the ischemic brains revealed that $IRG1^{-/-}$ MCAO mice exhibited a larger infarct volume than WT MCAO controls ($IRG1^{-/-}$ 55.0 ± 6.5 vs. WT 26.9 ± 4.0) (Fig. 1B bottom). The similar results were observed in female MCAO mice in which female $IRG1^{-/-}$ MCAO mice also developed a larger infarct than female WT MCAO controls ($IRG1^{-/-}$ 61.5 ± 7.4 vs. WT 26.4 ± 4.1) (Fig. S3). In addition, the long-term survival rate of WT and $IRG1^{-/-}$ MCAO mice was assessed. Our results showed that $IRG1^{-/-}$ MCAO mice displayed a decreased survival rate compared to WT MCAO controls in which *IRG1^{-/-}* MCAO mice had a survival rate of 50%, whereas WT MCAO controls had a survival rate of 70% at day 7 post-injury (Fig. 1C). Collectively, our results demonstrate that ischemic stroke induces IRG1 expression in the ischemic brain, however IRG1 deficiency exacerbates brain injury following ischemic insults.

IRG1 deficiency enhances neuroinflammation in ischemic stroke

To determine whether IRG1 deficiency-exacerbated ischemic brain injury is due to enhanced neuroinflammation, the ischemic brains were harvested from WT and $IRG1^{-/-}$ MCAO mice to assess MG activation. Mononuclear cells were isolated from the ischemic brains of WT and $IRG1^{-/-}$ MCAO mice and then subjected to FACS analysis to assess the expression of surface maturation markers, CD80 and CD86, on MG. MG were determined based on their intermediate expression of CD45 (CD45^{int}) and positive expression of CD11b (CD11b⁺). Although the level of CD80-expressing CD45^{int}CD11b⁺ cells in the ischemic brain was not altered between WT and $IRG1^{-/-}$ MCAO mice (data not shown), the number of CD86-expressing CD45^{int}CD11b⁺ cells in the ischemic brain was significantly increased in $IRG1^{-/-}$ MCAO mice compared to WT MCAO controls (Fig. 2A). In addition, ischemic brain tissues were harvested and subjected to IHC to assess the level of Iba1⁺ cells in the ipsilateral cortex and striatum of $IRG1^{-/-}$ MCAO mice was significantly higher than that in WT MCAO controls (Fig. 2B), confirming that IRG1 deficiency promotes MG activation in ischemic stroke.

As the peripheral immune cell infiltration of the ischemic brain has been reported to cause the secondary brain injury that in turn aggravates neuroinflammation, we therefore compared the level of peripheral immune cell infiltration in the ischemic brain of WT and $IRG1^{-/-}$ MCAO mice. Mononuclear cells were isolated from the ischemic brains of WT and $IRG1^{-/-}$ MCAO mice, and the CNS infiltrating immune cells were determined based on their high expression of CD45 (CD45^{hi}) and positive expression of CD11b (CD11b⁺). We did not observe increased CD45^{hi}CD11b⁺ cells in the ipsilateral hemisphere compared to the contralateral hemisphere in WT MCAO mice at 24 h post-injury. However, a significant increase of CD45^{hi}CD11b⁺ cells was observed in the ipsilateral hemisphere of $IRG1^{-/-}$ MCAO mice at 24 h post-injury (Fig. 2C), suggesting IRG1 deficiency promotes the immune cell infiltration of the CNS in ischemic stroke. Collectively, our results demonstrate that IRG1 deficiency promotes MG activation and immune cell infiltration that lead to enhanced neuroinflammation in ischemic stroke.

Deficiency in IRG1 results in decreased cerebral HO-1 expression following ischemic stroke

IRG1 induces the production of itaconate, and two itaconate derivatives, DMI and 4-octyl-itaconate, have been shown to induce Nrf2 activation and subsequent HO-1 upregulation (5, 8, 9). In addition, previous studies have demonstrated that the induction of HO-1 exerts a protective effect against ischemic brain injury (16, 18, 19). We therefore evaluated whether ischemic stroke induces HO-1 expression in the ischemic brain. Our results showed that HO-1 was highly upregulated in the ipsilateral but not contralateral hemisphere at both mRNA and protein levels in WT MCAO mice (Fig. 3A and B). To further investigate whether IRG1 deficiency affects cerebral HO-1 expression following ischemic stroke, we

compared the level of HO-1 expression in the ischemic brain between WT and $IRG1^{-/-}$ MCAO mice. We found that HO-1 expression was largely decreased in the ischemic brain of $IRG1^{-/-}$ MCAO mice compared to that in WT MCAO controls (Fig. 3C). As MG were reported to be the main producers of HO-1 (20), we then assessed whether MG produce HO-1 in the ischemic brain. We observed the co-localization of HO-1 and Iba1 immunoreactivity in the cortex and striatum of WT and $IRG1^{-/-}$ MCAO mice (Fig. 3D). In addition, we found that the level of HO-1 immunoreactivity was higher in the ischemic brain of WT MCAO mice than that of $IRG1^{-/-}$ MCAO mice, and that is consistent with our results of western blot analysis (Fig. 3C and D). Altogether, our results demonstrate that ischemic stroke induces HO-1 expression, and deficiency in IRG1 results in decreased HO-1 expression in the ischemic brain.

MG and MΦ deficient in IRG1 exhibit reduced HO-1 expression following LPS challenge

To confirm our in vivo findings that IRG1 deficiency decreases cerebral HO-1 expression following ischemic stroke, MG and M Φ were generated from WT and *IRG1^{-/-}* mice and then stimulated with LPS to assess IRG1 and HO-1 expression. At the mRNA level, IRG1 was highly upregulated at 5 h and declined at 8 h after LPS stimulation in both WT MG and M Φ (Fig. 4A). At the protein level, IRG1 was slightly induced at 8 h and strongly upregulated at 16 h after LPS stimulation in both MG and M Φ (Fig. 4A). At the protein level, IRG1 was slightly induced at 8 h and strongly upregulated at 16 h after LPS stimulation in both MG and M Φ (Fig. 4B left panel). However, IRG1 protein expression was not observed in *IRG1^{-/-}* MG and M Φ after LPS stimulation (Fig. 4B right panel). We then compared HO-1 mRNA expression in WT and *IRG1^{-/-}* MG and M Φ . Although the basal level of HO-1 mRNA expression was downregulated by LPS treatment at the early time points (5 h and 8 h after LPS treatment) in both WT and *IRG1^{-/-}* MG, LPS induced a strong upregulation of HO-1 only in WT MG but not in *IRG1^{-/-}* MG at the late time point (24 h after LPS treatment) (Fig. 4C). The similar results were observed in M Φ . Although there was a slight upregulation of HO-1 in *IRG1^{-/-}* M Φ after 24 h LPS treatment, the level of HO-1 expression was significantly lower in *IRG1^{-/-}* M Φ compared to that in WT M Φ (Fig. 4D). Collectively, our results demonstrate that IRG1 induces HO-1 expression, and deficiency in IRG1 results in reduced HO-1 expression in MG and M Φ following inflammatory stimuli.

IRG1 deficiency represses BDNF expression in the ischemic brain, however IRG1 deficiency-induced BDNF repression and ischemic brain injury exacerbation can be rescued by D3T

HO-1 induction has been shown to attenuate ischemic stroke induced-hippocampal neuronal injury via the activation of BDNF-TrkB-PI3K/Akt signaling pathway (21). Our observation of decreased HO-1 expression in the ischemic brain of $IRG1^{-/-}$ MCAO mice prompted us to investigate whether IRG1 deficiency may lead to repressed BDNF expression in the ischemic brain. Indeed, we found that BDNF expression at both mRNA and protein levels were repressed in the ischemic brain of $IRG1^{-/-}$ MCAO mice

compared to that in WT MCAO controls (Fig. 5A and C). To further evaluate whether IRG1 deficiencyinduced BDNF repression can be rescued by the induction of HO-1 expression, *IRG1^{-/-}* MCAO mice were administered with either vehicle or D3T, an Nrf2/HO-1 pathway inducer (16, 22–24), and the ischemic brains were then harvested to assess BDNF expression. D3T strongly upregulated BDNF expression in the ischemic brain of *IRG1^{-/-}* MCAO mice at both mRNA and protein levels (Fig. 5B and C). Strikingly, IRG1 deficiency-exacerbated ischemic brain injury was lessened by D3T treatment in *IRG1^{-/-}* MCAO mice. Although the physiological parameters of vehicle- and D3T-treated *IRG1^{-/-}* stroke animlas during MCAO were comparable (Table S2), the infarct volume of D3T-treated *IRG1^{-/-}* MCAO mice was significantly lower than that of vehicle-treated *IRG1^{-/-}* MCAO mice (D3T 24.9 ± 2.7 vs. Vehicle 55.6 ± 7.1) (Fig. 5D). Altogether, our results demonstrate that IRG1 deficiency represses BDNF expression in the ischemic brain following ischemic stroke, and D3T, an Nrf2/HO-1 pathway inducer, is able to enhance BDNF expression and lessen ischemic brain injury in *IRG1^{-/-}* stroke animals.

Discussion

IRG1, a mitochondrial enzyme, mediates the production of itaconate during inflammatory responses in myeloid lineage cells (25). Recent studies revealed the unique function of IRG1/itaconate axis in modulating inflammation and further demonstrated the anti-inflammatory effects of itaconate on the suppression of succinate dehydrogenase (SDH) activities, activation of anti-oxidant and antiinflammatory Nrf2/HO-1 pathways, and amelioration of disease severity in the psoriasis animal model (8, 9, 19). Presently, the immunomodulatory effects of IRG1/itaconate axis were mainly evaluated in the peripheral inflammatory immune responses. Although we have recently reported that DMI, an itaconate derivative, suppressed neuroinflammation and ameliorated disease severity in the chronic CNS disease, EAE (12), whether IRG1/itaconate axis modulates the acute CNS disease, ischemic stroke, remains unexplored. Hence, in this study we investigated the potential immunomodulatory effects of IRG1 on the alleviation of ischemic brain injury and elucidated the molecular mechanisms underlying the protective effect of IRG1 in ischemic stroke. We observed that IRG1 was highly induced in the injured brain following ischemic insults. Importantly, we found that $IRG1^{-/-}$ stroke animals developed a much severe brain injury compared to WT stroke controls. Thus, we identify a novel function of IRG1 in the acute CNS disease and demonstrate that the induction of IRG1 following ischemic stroke may serve as an endogenous protective mechanism to restrain ischemic brain injury.

Neuroinflammation plays a pivotal role in brain injury following ischemic stroke. MG activation and peripheral immune cell infiltration have been shown to contribute to the induction and aggravation of neuroinflammation in ischemic stroke (14, 16). Since we observed exacerbated ischemic brain injury in $IRG1^{-/-}$ stroke animals, we speculate that IRG1 deficiency may promote neuroinflammation in ischemic stroke. Indeed, we observed increased CD86-expressing MG as well as elevated Iba1⁺ cells in the ischemic brain of $IRG^{-/-}$ stroke animals, indicating IRG1 deficiency enhances MG activation. In addition, we found that increased peripheral immune cells infiltrate into the ischemic brain of $IRG1^{-/-}$ stroke animals when compared to that of WT stroke controls. Collectively, our findings suggest that one of the protective

mechanisms of IRG1 in ischemic stroke may be mediated through its's immunomodulatory effect on the suppression of MG activation and inhibition of peripheral immune cell infiltration.

Previous studies demonstrate that the induction of HO-1 exerts a protective effect against ischemic stroke (16, 26–29). In addition, a study shows that HO-1 plays the essential role in ischemic preconditioning (IPC)-induced protection against brain ischemia, as IPC fails to protect HO-1 deficient mice against permanent ischemic brain injury (30). In this study, we found that both IRG1 and HO-1 were highly induced in the ipsilateral but not the contralateral hemisphere of WT stroke animals. Most importantly, we observed HO-1 expression was largely decreased in the ischemic brain of *IRG1^{-/-}* stroke animals compared to that of WT stroke controls. These observations correlate with our results showing *IRG1^{-/-}* MCAO mice developed a much severe brain injury than WT MCAO controls, and further confirm the protective effect of HO-1 in ischemic stroke. In addition, to verify the essential role of IRG1 in the induction of endogenous HO-1 expression, primary MG and MΦ were generated from WT and *IRG1^{-/-}* mice to determine HO-1 expression. We found *IRG1^{-/-}* MG stimulated with LPS expressed a very lower level of HO-1 compared to WT MG stimulate with LPS, and the similar results were also observed in MΦ. Thus, our results demonstrate that IRG1 is required for endogenous HO-1 expression following inflammatory stimuli in vitro and in vivo, and suggest that the induction of IRG1 following ischemic stroke promotes HO-1 expression to restrain ischemic brain injury.

BDNF participates in neuronal plasticity and promotes neuronal survival and growth (31). Interestingly, we found that following ischemic stroke the cerebral BDNF expression at both mRNA and protein levels were significantly lower in $IRG1^{-/-}$ stroke animals compared to WT stroke controls. As HO-1 has been shown to promote BDNF expression (21), we speculate that IRG1 deficiency-induced HO-1 reduction may be responsible for the repressed BDNF expression in the ischemic brain of $IRG1^{-/-}$ stroke animals. To test our hypothesis, we thought to enhance BDNF expression in $IRG1^{-/-}$ stroke animals by administration of D3T, an Nrf2/HO-1 pathway inducer. Indeed, D3T treatment enhanced BDNF expression in the ischemic brain injury in $IRG1^{-/-}$ stroke animals. Thus, our results demonstrate that IRG1 deficiency-induced BDNF repression and ischemic brain injury exacerbation can be rescued by D3T, and that may be mediated through the effect of D3T on the induction of HO-1 expression in the ischemic brain.

During respiration with normal oxygen consumption, SDH functions to break down succinate in the TCA cycle. During ischemia, SDH works reversely that leads to succinate accumulation. Indeed, a high level of succinate can be detected in the ischemic tissues of heart, live, kidney and brain (32). The accumulated succinate can then be rapidly oxidized following reperfusion that results in increased mitochondrial ROS production. Notably, the inhibition of SDH activity was reported to offer a protection against ischemic injury (32, 33). As IRG1/itaconate axis has been shown to suppress SDH activity (8, 34), further studies would be required to investigate whether IRG1 deficiency-exacerbated ischemic brain injury may be partly due to elevated succinate accumulation and increased ROS production in the ischemic brain. In addition, as IRG1 has been shown to induce A20 expression to elicit anti-inflammatory effects (6, 35, 36), it would

be important to further investigate whether IRG1-inudced A20 expression plays a role in modulating ischemic brain injury.

Conclusions

In this study, we report for the first time that the induction of IRG1 in the ischemic brain following ischemic stroke represents an endogenous protective mechanism to restrain ischemic brain injury. We identify that IRG1 deficiency exacerbates ischemic brain injury exhibited with elevated MG activation and increased peripheral immune cell infiltration in the ischemic brain. Most importantly, we demonstrate that IRG1 is required for optimal cerebral HO-1 and BDNF expression following ischemic stroke, as the expression of cerebral HO-1 and BDNF was repressed in $IRG1^{-/-}$ stroke animals compared to that in WT stroke controls. We further demonstrate that the repressed expression of cerebral HO-1 and BDNF may be responsible for exacerbated ischemic brain injury in $IRG1^{-/-}$ stroke animals, as the administration of D3T, an Nrf2/HO-1 pathway inducer, induces cerebral BDNF expression and lessens ischemic brain injury in $IRG1^{-/-}$ stroke animals (Fig. 6). In summary, our study reveals a novel function of IRG1 in modulating ischemic brain injury through the induction of cerebral HO-1 and BDNF expression following ischemic brain injury is strategy for the treatment of ischemic stroke.

Abbreviations

IRG1: immunoresponsive gene 1; MCAO: middle cerebral artery occlusion (MCAO); MG: microglia; DMI: dimethyl itaconate; TTC: triphenyltetrazolium chloride; CBF: cerebral blood flow; Q-PCR: quantitative PCR; WT: wild type; SDH: succinate dehydrogenase; ROS: reactive oxygen species.

Declarations

Funding: This work was supported in part by NIH R01NS102449 to J-H. Yen.

Availability of data and materials: The datasets of the current study are available from the corresponding author on a reasonable request.

Author's contributions: PCK performed the experiments, analyzed data and wrote the manuscript. WTW performed the experiments and analyzed data. BAS, DF and HCP performed the experiments. ICY reviewed and edited the manuscript. JHY conceived the study, designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements: Not applicable.

Ethics approval and consent to participate: All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This study does not contain any experiments with human participants performed by any of the authors.

Consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing interests.

References

- 1. Kunz A, Dirnagl U, Mergenthaler P. Acute pathophysiological processes after ischaemic and traumatic brain injury. Best Pract Res Clin Anaesthesiol. 2010;24(4):495–509.
- Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation. 2017;135(10):e146–603.
- 3. Planas AM. Role of Immune Cells Migrating to the Ischemic Brain. Stroke. 2018;49(9):2261-7.
- 4. Jayaraj RL, Azimullah S, Beiram R, Jalal FY, Rosenberg GA. Neuroinflammation: friend and foe for ischemic stroke. J Neuroinflammation. 2019;16(1):142.
- 5. Mills EL, Ryan DG, Prag HA, Dikovskaya D, Menon D, Zaslona Z, et al. Itaconate is an antiinflammatory metabolite that activates Nrf2 via alkylation of KEAP1. Nature. 2018;556(7699):113–7.
- Li Y, Zhang P, Wang C, Han C, Meng J, Liu X, et al. Immune responsive gene 1 (IRG1) promotes endotoxin tolerance by increasing A20 expression in macrophages through reactive oxygen species. J Biol Chem. 2013;288(23):16225–34.
- 7. Dominguez-Andres J, Novakovic B, Li Y, Scicluna BP, Gresnigt MS, Arts RJW, et al. The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity. Cell Metab. 2019;29(1):211–20. e5.
- Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, Loginicheva E, et al. Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. Cell Metab. 2016;24(1):158–66.
- 9. Bambouskova M, Gorvel L, Lampropoulou V, Sergushichev A, Loginicheva E, Johnson K, et al. Electrophilic properties of itaconate and derivatives regulate the IkappaBzeta-ATF3 inflammatory axis. Nature. 2018;556(7702):501–4.
- 10. Cordes T, Lucas A, Divakaruni AS, Murphy AN, Cabrales P, Metallo CM. Itaconate modulates tricarboxylic acid and redox metabolism to mitigate reperfusion injury. Mol Metab. 2020;32:122–35.
- 11. Zhang D, Lu Z, Zhang Z, Man J, Guo R, Liu C, et al. A likely protective effect of dimethyl itaconate on cerebral ischemia/reperfusion injury. Int Immunopharmacol. 2019;77:105924.
- 12. Kuo PC, Weng WT, Scofield BA, Paraiso HC, Brown DA, Wang PY, et al. Dimethyl itaconate, an itaconate derivative, exhibits immunomodulatory effects on neuroinflammation in experimental autoimmune encephalomyelitis. J Neuroinflammation. 2020;17(1):138.
- 13. Kuo PC, Brown DA, Scofield BA, Yu IC, Chang FL, Wang PY, et al. 3H-1,2-dithiole-3-thione as a novel therapeutic agent for the treatment of experimental autoimmune encephalomyelitis. Brain Behav Immun. 2016;57:173–86.

- 14. Kuo PC, Scofield BA, Yu IC, Chang FL, Ganea D, Yen JH. Interferon-beta Modulates Inflammatory Response in Cerebral Ischemia. J Am Heart Assoc. 2016;5(1).
- Hooper KM, Yen JH, Kong W, Rahbari KM, Kuo PC, Gamero AM, et al. Prostaglandin E2 Inhibition of IL-27 Production in Murine Dendritic Cells: A Novel Mechanism That Involves IRF1. J Immunol. 2017;198(4):1521–30.
- 16. Kuo PC, Yu IC, Scofield BA, Brown DA, Curfman ET, Paraiso HC, et al. 3H-1,2-Dithiole-3-thione as a novel therapeutic agent for the treatment of ischemic stroke through Nrf2 defense pathway. Brain Behav Immun. 2017;62:180–92.
- 17. Yen JH, Khayrullina T, Ganea D. PGE2-induced metalloproteinase-9 is essential for dendritic cell migration. Blood. 2008;111(1):260–70.
- Cen J, Zhao N, Huang WW, Liu L, Xie YY, Gan Y, et al. Polyamine analogue QMA attenuated ischemic injury in MCAO rats via ERK and Akt activated Nrf2/HO-1 signaling pathway. Eur J Pharmacol. 2019;844:165–74.
- 19. Hu L, Chen W, Tian F, Yuan C, Wang H, Yue H. Neuroprotective role of fucoxanthin against cerebral ischemic/reperfusion injury through activation of Nrf2/HO-1 signaling. Biomed Pharmacother. 2018;106:1484–9.
- 20. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci. 2014;34(36):11929–47.
- 21. Qi D, Ouyang C, Wang Y, Zhang S, Ma X, Song Y, et al. HO-1 attenuates hippocampal neurons injury via the activation of BDNF-TrkB-PI3K/Akt signaling pathway in stroke. Brain Res. 2014;1577:69–76.
- 22. Li KR, Yang SQ, Gong YQ, Yang H, Li XM, Zhao YX, et al. 3H-1,2-dithiole-3-thione protects retinal pigment epithelium cells against Ultra-violet radiation via activation of Akt-mTORC1-dependent Nrf2-HO-1 signaling. Sci Rep. 2016;6:25525.
- 23. Cui Y, Ma S, Zhang C, Li D, Yang B, Lv P, et al. Pharmacological activation of the Nrf2 pathway by 3H-1, 2-dithiole-3-thione is neuroprotective in a mouse model of Alzheimer disease. Behav Brain Res. 2018;336:219–26.
- 24. Ghosh S, Hazra R, Ihunnah CA, Weidert F, Flage B, Ofori-Acquah SF. Augmented NRF2 activation protects adult sickle mice from lethal acute chest syndrome. Br J Haematol. 2018;182(2):271–5.
- 25. Michelucci A, Cordes T, Ghelfi J, Pailot A, Reiling N, Goldmann O, et al. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. Proc Natl Acad Sci U S A. 2013;110(19):7820–5.
- 26. Saleem S, Zhuang H, Biswal S, Christen Y, Dore S. Ginkgo biloba extract neuroprotective action is dependent on heme oxygenase 1 in ischemic reperfusion brain injury. Stroke. 2008;39(12):3389–96.
- 27. Chen Y, Zhang X, Yang Y, Zhang L, Cui L, Zhang C, et al. Tert-butylhydroquinone enhanced angiogenesis and astrocyte activation by activating nuclear factor-E2-related factor 2/heme oxygenase-1 after focal cerebral ischemia in mice. Microvasc Res. 2019;126:103891.

- 28. Wang P, Zhao Y, Li Y, Wu J, Yu S, Zhu J, et al. Sestrin2 overexpression attenuates focal cerebral ischemic injury in rat by increasing Nrf2/HO-1 pathway-mediated angiogenesis. Neuroscience. 2019;410:140–9.
- 29. Wei CC, Kong YY, Li GQ, Guan YF, Wang P, Miao CY. Nicotinamide mononucleotide attenuates brain injury after intracerebral hemorrhage by activating Nrf2/HO-1 signaling pathway. Sci Rep. 2017;7(1):717.
- 30. Zeynalov E, Shah ZA, Li RC, Dore S. Heme oxygenase 1 is associated with ischemic preconditioninginduced protection against brain ischemia. Neurobiol Dis. 2009;35(2):264–9.
- 31. Lipsky RH, Marini AM. Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. Ann N Y Acad Sci. 2007;1122:130–43.
- 32. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature. 2014;515(7527):431–5.
- 33. Wu B, Luo H, Zhou X, Cheng CY, Lin L, Liu BL, et al. Succinate-induced neuronal mitochondrial fission and hexokinase II malfunction in ischemic stroke: Therapeutical effects of kaempferol. Biochim Biophys Acta Mol Basis Dis. 2017;1863(9):2307–18.
- 34. Cordes T, Wallace M, Michelucci A, Divakaruni AS, Sapcariu SC, Sousa C, et al. Immunoresponsive Gene 1 and Itaconate Inhibit Succinate Dehydrogenase to Modulate Intracellular Succinate Levels. J Biol Chem. 2016;291(27):14274–84.
- 35. Jamal Uddin M, Joe Y, Kim SK, Oh Jeong S, Ryter SW, Pae HO, et al. IRG1 induced by heme oxygenase-1/carbon monoxide inhibits LPS-mediated sepsis and pro-inflammatory cytokine production. Cell Mol Immunol. 2016;13(2):170–9.
- 36. Van Quickelberghe E, Martens A, Goeminne LJE, Clement L, van Loo G, Gevaert K. Identification of Immune-Responsive Gene 1 (IRG1) as a Target of A20. J Proteome Res. 2018;17(6):2182–91.

Figures



Figure 1

Ischemic stroke induces IRG1 expression in the ischemic brain, however IRG1 deficiency exacerbates brain injury following ischemic insults. (A) C57BL/6 mice were subjected to 40 minutes MCAO (n=8), and the ischemic brains were harvested at 6 hours post-reperfusion. The contralateral (cont.) and ipsilateral (ipsi.) hemisphere of the ischemic brains were separated and subjected to Q-PCR analysis for Irg1 expression. **p<0.01 by unpaired t test. (B) WT and IRG1-/- mice were subjected to MCAO (n=10/group). 24 hours after injury, mice were sacrificed, and ischemic brains were harvested and sliced (2 mm)

followed by TTC staining. Three representative TTC-stained samples of WT and IRG1-/- MCAO mice were shown. The infarct volumes were also measured. **p<0.01 by unpaired t test. (C) The Kaplan-Meier survival curve of WT and IRG1-/- MCAO mice (n=10/group) was evaluated up to day 7 post-injury.



Figure 2

IRG1 deficiency enhances neuroinflammation in ischemic stroke. WT and IRG1-/- mice were subjected to 40 minutes MCAO. (A) At 15h post-injury, the ischemic brains were harvested from WT and IRG1-/- MCAO

mice and separated into the contralateral (cont.) and ipsilateral (ipsi.) hemisphere followed by mononuclear cell isolation (n=4-5/group). The isolated cells were stained with antibodies of CD45, CD11b and CD86. MG were determined based on their expression of CD45intCD11b+, and CD45intCD11b+ cells with positive expression of CD86 were then determined. Isotype controls (ISO) were used as a negative control to determine MG positive for CD86 expression. **p<0.05; ***p<0.001 by one way ANOVA. (B) At 24 hours post-injury, the ischemic brains were harvested from WT and IRG1-/- MCAO mice followed by IHC analysis to determine lba1 expression (n=4-5/group). The representative images of lba1 immunostaining in the ipsilateral cortex and striatum of peri-infarct regions were shown. lba1+ cells per mm2 were also quantified. Scale bar: 20 µm. *p<0.05 by unpaired t test. (C) Mononuclear cells were isolated from the contralateral (cont.) and ipsilateral (ipsi.) hemisphere of WT and IRG1-/- MCAO mice (n=8/group). The isolated cells were then stained with antibodies against CD11b and CD45 followed by FACS analysis. The CNS infiltrating immune cells were determined based on the expression of CD45hiCD11b+. **p<0.01; N.S., no significant differences by one way ANOVA.

Figure 3



Figure 3

Deficiency in IRG1 results in decreased cerebral HO-1 expression following ischemic stroke. C57BL/6 were subjected to 40 minutes MCAO. (A) At 6 hours post-reperfusion, the ischemic brains were harvested and separated into the contralateral (cont.) and ipsilateral (ipsi.) hemisphere followed by Q-PCR analysis for HO-1 expression (n=8). ***p<0.001 by unpaired t test. (B) At 24 hours post-injury, the contralateral (cont.) and ipsilateral (ipsi.) hemisphere followed by D-PCR analysis for HO-1 expression (n=8). ***p<0.001 by unpaired t test. (B) At 24 hours post-injury, the contralateral (cont.) and ipsilateral (ipsi.) hemisphere tissues were subjected to western blot analysis for HO-1

expression. Two representative results of HO-1 expression in the contralateral and ipsilateral hemisphere tissues were shown. (C) The ischemic brains harvested from WT and IRG1-/- MCAO mice at 24 hours post-injury were separated into the contralateral and ipsilateral hemisphere, and the ipsilateral hemisphere tissues were then subjected to western blot analysis for HO-1 expression. The level of HO-1 expression was also quantified (n=6/group). **p<0.01 by unpaired t test. (D) The ischemic brains of WT and IRG1-/- MCAO mice were subjected to IHC analysis to determine Iba1 and HO-1 co-localization. The representative images of HO-1 and Iba1 expression in the ipsilateral cortex and striatum of peri-infarct regions were shown (n=4-5/group). Scale bar: 20 µm; 5 µm in magnified boxes.

Figure 4



Figure 4

MG and MΦ deficient in IRG1 exhibit reduced HO-1 expression following LPS challenge. (A) Primary MG and MΦ were stimulated with LPS 100 ng/ml for 5 hours and 8 hours followed by Q-PCR analysis for IRG1 expression. ***p<0.001 by one way ANOVA. (B) Primary MG and MΦ generated from WT or IRG1-/- mice were stimulated with LPS 100 ng/ml for a time course followed by western blot analysis for IRG1 expression. (C) Primary MG and (D) MΦ generated from WT or IRG1-/- mice were treated with LPS 100

ng/ml for a time course and then subjected to Q-PCR analysis for HO-1 expression. The representative results of 3-4 independent experiments were shown. ***p<0.001 by one way ANOVA.

Figure 5



Figure 5

IRG1 deficiency represses BDNF expression in the ischemic brain, however IRG1 deficiency-induced BDNF repression and ischemic brain injury exacerbation can be rescued by D3T. WT and IRG1-/- mice were subjected to 40 minutes MCAO. (A) The ipsilateral hemispheres of WT and IRG1-/- MCAO mice were

harvested at 24 hours post-injury followed by Q-PCR analysis for BDNF expression (n=6/group). ***p<0.001 by unpaired t test. (B-D) IRG1-/- mice subjected to 40 minutes MCAO were i.p. administered with vehicle or D3T (50 mg/kg) at 90 minutes post-reperfusion. (B) At 24 hours post-injury, the ipsilateral hemispheres of IRG1-/- MCAO mice administered with vehicle or D3T were harvested and subjected to Q-PCR analysis for BDNF expression (n=6/group). ***p<0.001 by unpaired t test. (C) The ipsilateral hemispheres of WT MCAO mice as well as vehicle- and D3T-treated IRG1-/- MCAO mice were harvested and subjected to western blot analysis to determine BDNF expression. Three representative images of BDNF expression from each group were shown, and the level of BDNF expression was also quantified (n=9/group). *p<0.05; **p<0.01 by one way ANOVA. (D) At 24 hours post-injury, the ischemic brains harvested from vehicle- and D3T-treated IRG1-/- MCAO mice were subjected to TTC staining. Three representative TTC-stained samples from each group were shown. The infarct volume of vehicle- and D3T-treated IRG1-/- MCAO mice was also measured (n=10/group). **p<0.01 by unpaired t test.



Figure 6

Figure 6

The model of IRG1-mediated HO-1 and BDNF induction in modulating ischemic brain injury. (A) Ischemic stroke induces IRG1 expression in the ischemic brain that promotes cerebral HO-1 and BDNF expression to restrain ischemic brain injury. (B) Deficiency in IRG1 results in repressed HO-1 and BNDF expression in the ischemic brain, leading to exacerbated ischemic brain injury. (C) D3T, an Nrf2/HO-1 pathway inducer, induces BDNF expression and lessens ischemic brain injury in IRG1-/- stroke animals.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

• IRG1KOStrokeSupplementalFinal.pdf