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Oxygen and glucose deprivation induces expression of Aim2, ASC, caspase-1 and NLRP3 in primary microglia and bone marrow-derived macrophages

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Abstract

Microglia/macrophages are recognized as major contributors to brain inflammation after an ischemic stroke, which is metabolically characterized by oxygen and glucose deprivation (OGD). The impact of OGD on the expression of inflammasome-related proteins within microglia/macrophages remains unclear. Herein we report that OGD upregulates the expression of Aim2, ASC, caspase-1, NRLP3 and ultimately IL-1β in microglia and bone marrow-derived macrophages (BMDM).

Introduction

Each year ~795,000 people within the United States experience stroke events, and of these, 87% are ischemic [1]. The governing pathobiology of ischemic brain stroke initially unfolds due to a focal deficit in metabolism driven primarily by oxygen and glucose deprivation (OGD) [2]. Recently, inflammation and immune responses have emerged as important elements in both the onset and progression of stroke and have come to be recognized as primary contributors to ischemic brain injury [3]. Of particular interest are microglia within the brain and macrophages from the periphery that have both been shown to have prominent roles in initiating, sustaining, and resolving post-ischemic inflammation and, therefore, represent attractive therapeutic targets [4]. Microglia-the resident immune cells of the central nervous system (CNS)-retract their processes and in doing so display an amoeboid-like morphology soon after the onset of ischemic stroke; these activated cells are morphologically indistinguishable from activated macrophages [5]. As per the abovementioned, blood-derived monocytes rapidly infiltrate into the brain and become macrophages following brain ischemia [6] [7]. Activated microglia/macrophages release pro-inflammatory cytokines that lead to the development of secondary injuries [6] [8] [5].

Inflammasomes are multi-protein complexes that act as both sensors of host-derived danger signals and/or infectious agents. As such, they play important roles in mediating inflammation in sepsis, metabolic/autoimmune disorders, cancer, and ischemic/reperfusion-associated injuries [9] [10] [11]. Inflammasomes trigger the maturation of caspase-1 followed by the production of IL-1β, which promotes inflammation and cell death in multiple diseases/disorders [12] [13] [14].

IL-1β expression levels within the brain increase following ischemia in experimental stroke [15] and have also been shown to increase in both the cerebrospinal fluid and blood of post-stroke patients [16] [17]. In addition, multiple studies have demonstrated that targeting IL-1β may be neuroprotective in animal models of ischemic brain injury [8]. It is critical to note that various studies have also indicated that microglia/macrophages serve as the major sources of IL-1β within the ischemic brain [15] [8]. In animal models, the levels of inflammasome-related proteins increase after brain ischemia and the inhibition of inflammasome activity has been shown to be capable of reducing the extent of injury [18] [19] [20] [21].

Despite the defined links between ischemia and innate inflammation, the impact of OGD on the expression of inflammasome-related proteins and ultimately IL-1β in microglia/macrophages remains to be elucidated. Given the role that microglia/macrophages play in the development/progression of brain injury after ischemia, herein we sought to examine the effects of OGD on inflammasome-related

protein expression and the modulation of IL-1β activation/release within these cells. We found that OGD rapidly induced Aim2 (absent in melanoma 2), ASC (apoptosis-associated speck-like protein containing a CARD), caspase-1, and NLRP3 (NLR family, pyrin domain containing 3) expression and boosted the LPS-induced production of IL-1β in microglia and bone marrow-derived macrophages (BMDM).

Objective

To determine the influence of OGD on the expression of inflammasome-related proteins/IL-1β within primary microglia and macrophages.

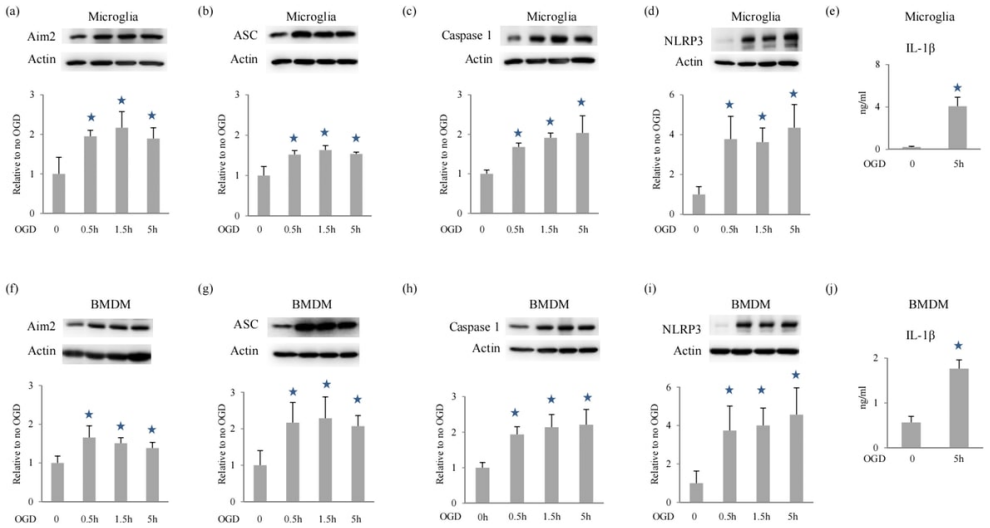

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Figure Legend

Figure 1. Oxygen and glucose deprivation (OGD) upregulates Aim2, ASC, caspase-1, NLRP3, and the release of the pro-inflammatory cytokine IL-1β in primary microglia and bone marrow-derived macrophages (BMDM).

(A-D) Primary microglia cells were subjected to OGD for 0.5, 1.5, and 5 h. Aim2, ASC, caspase-1 and NLRP3 expression were evaluated via western blotting.

(E) Primary microglia cells were subjected to OGD and 10 ng/ml LPS for 5 h. Cell culture supernatants were collected and cytokine levels were measured via ELISA.

(F-I) BMDM were subjected to OGD for 0.5, 1.5, and 5 h. Aim2, ASC, caspase-1, and NLRP3 expression were evaluated via western blotting.

(J) BMDM cells were subjected to OGD and 10 ng/ml LPS for 5 h. Cell culture supernatants were collected and cytokine levels were measured via ELISA. All data are presented as mean ± SD of three independent experiments.

Reagents/kits

Rat macrophage colony stimulating factor (M-CSF) was purchased from GenScript (Piscataway, NJ). ELISA kits were obtained from R&D Systems (Minneapolis, MN). Lipopolysaccharide (LPS) from *Escherichia coli* OIII:B4 was purchased from Invivogen (San Diego, CA; #LPS-EB). All assays and techniques were performed according to the provided guidelines.

Spontaneously Hypertensive Stroke Prone (SHR-SP) and Sprague Dawley (SD) rats

Male and female SHR-SP rats were used to produce the BMDM. Timed pregnant SD rats were used in the production of microglia and were purchased from Taconic (Hudson, NY).

Bone marrow derived macrophages

BMDM were derived from the femurs/tibias of SHR-SP rats as has been previously described [32]. DMEM supplemented with 10% FBS and 10 ng/ml rat M-CSF was used to culture the cells. Briefly, a 100 mm tissue culture dish was seeded with 2×10^6 cells in a total of 11 ml culture medium. After 3 days, 5.5 ml medium with 30 ng/ml rat M-CSF was added to each dish. After 6 days in total, PBS was used to wash the cells twice prior to scraping in cold HBSS. After centrifugation and resuspension, the cell density was adjusted to 2.5×10^5 cells per ml and 2 ml cells were deposited in each well of six-well-plates. Iba-1 staining confirmed that >99% of the cells were macrophages. On the 7^{th} day, the cells were exposed to OGD or OGD + LPS (10 ng/ml). Supernatants from OGD/LPS-treated cells were collected and used for ELISA.

Primary microglia

The isolation of cells was performed as previously described [33] with minor modifications. Briefly, 2–4 day old pups (determined via the use of timed pregnant SD rats) were used to make primary microglia. After the brains were dissected, the meninges were removed and the cortices placed in cold HBSS. After treatment with papain and DNase (Worthington Biochemical, Lakewood, NJ) for 15 min at 37℃, the cells were triturated 10x followed by centrifugation at 300 *g* for 5 min. The cells were then resuspended in DMEM plus 10% FBS and 10% horse serum (Life Technologies, Frederick, MD) and seeded at 2.5×10^5 cells per ml in poly-L-lysine (Sigma, St. Louis, MO) coated flasks; the medium was changed the following day. Microglia within the supernatant were collected 4–6 days later, plated on poly-L-lysine coated plates, and used experimentally on the following day.

Oxygen and glucose deprivation

Prior to OGD cells were washed twice with PBS and 1 ml culture medium without glucose (Life Technologies, Grand Island, NY), 1 ml of the glucose-free media was subsequently added to each well. The plates were then placed within modular incubator chambers (Billups-Rothenberg, San Diego, CA) with an anaerobic indicator strip capable of detecting a 0.2% oxygen threshold (Becton Dickinson). The chamber was flushed with a gas mixture containing 95% N_2 and 5% CO_2 at 6 L/min for 20 min at room temperature, sealed and placed at 37℃.

Western blots

Commercial IP lysis buffer (Thermo Fisher Scientific, Rockford, IL) was added directly to each well of the six-well plates to prepare cell lysates. The lysate was incubated for 15 min on ice and subsequently underwent 5 s of sonication. The lysate was then centrifuged at 10,000 *g* for 15 min at 4℃. The supernatant was collected and a BCA assay (Thermo Fisher Scientific, Rockford, IL) was used to determine the protein concentration. The samples were then heated for 5 min at 95℃, and 15 μg of the total cell lysate was used for SDS-PAGE. The following primary antibodies were used for the western blot analyses: anti-caspase-1 (Abcam, Cambridge, MA; #ab108362), anti-NLRP3 (AdipoGen, San Diego, CA; #AG-168 20B-0014), anti-ASC (AdipoGen, San Diego, CA; #AG-25B-0006), anti-AIM2 (Santa Cruz Biotechnology, Santa Cruz, CA; #SC137967). Signals were detected using a chemiluminescent substrate (Immobilon Western; Millipore, Billerica, MA) followed by digital imaging (Alpha Innotech, San Leandro, CA) or C-Digit (LI-COR, Lincoln, NE).

Statistics

All data are expressed as mean ± SD of three independent experiments. Western blot and ELISA data were compared using the Student's t-test. Differences were considered significant when p <0.05.

Results & Discussion

OGD upregulated Aim2, ASC, caspase-1, and NLRP3 protein levels in primary microglia

After 0.5 h, OGD upregulated Aim2, ASC, caspase-1, and NLRP3 protein levels (Fig. 1A-D), ranging from 1.5 ± 0.1-fold for ASC to 3.8 ± 1.1-fold for NLRP3. Aim2 was increased to 1.9 ± 0.2-fold and caspase-1 was increased to 1.7 ± 0.1-fold. Of note, the increased expression of these proteins was maintained for at least 5 h after OGD.

OGD and LPS (10 ng/ml) upregulated IL-1β in primary microglia

In microglia treated with OGD/LPS, IL-1β increased from 0.2 ± 0.07 to 4.1 ± 0.8 ng/ml after 5 h (Fig. 1E).

OGD upregulated Aim2, ASC, caspase-1, and NLRP3 protein levels in BMDM

Similar to microglia, OGD upregulated Aim2 (1.6 ± 0.3-fold), ASC (2.2 ± 0.5-fold), caspase-1 (1.9 ± 0.2-fold), and NLRP3 (3.7 ± 1.3-fold) protein levels in BMDM after 0.5 h OGD, and the increased levels of the proteins noted were maintained at 5 h after OGD (Fig. 1F-I).

OGD and LPS (10 ng/ml) upregulated IL-1β in BMDM

In BMDM treated with OGD/LPS, IL-1β increased from 0.6 ± 0.1 to 1.8 ± 0.2 ng/ml after 5 h (Fig. 1I).

Ischemic stroke is characterized by the interruption of cerebral blood flow, which results in severe tissue damage and/or death; its increasing prevalence continues to position stroke as a major public health burden throughout the world. As such, the elucidation of pertinent molecular details governing the response of inflammatory cells after exposure to ischemia may ultimately define how these cells contribute to ischemic brain injury. Herein we demonstrate that both microglia and macrophages significantly increase the expression levels of Aim2, ASC, caspase-1, NLRP3, and ultimately IL-1β production in a response to OGD or OGD/LPS.

Inflammasomes are essential in mediating inflammation in infection, ischemia/reperfusion injuries, metabolic and autoimmune disorders, and cancer as they act as sensors of both host-derived danger signals and infectious agents [10] [9]. While inflammasomes have all been implicated in experimental stroke, the precise cellular and molecular mechanisms remain to be elucidated. It is interesting to note that evidence has recently emerged to link genetic variants of NLRP3 with stroke in humans [22]. We found that NLRP3 was unregulated in both microglia and macrophages subjected to OGD. This finding is consistent with existing evidence that supports the activation of the NLRP3 inflammasome via exposure to extracellular ATP, reactive oxygen species, and/or necrotic cell death [23] [24], all of which are upregulated after stroke [6]. Further, it has been reported that brain ischemia causes oxidative DNA damage/fragmentation following experimental stroke [25] and that Aim2 senses cytoplasmic double-stranded DNA and in doing so activates the inflammasome [26] this is in perfect alignment with our results that demonstrate that Aim2 is unregulated in both microglia and macrophages that have been subjected to OGD. Our work also supports a recent finding that the number of activated microglia within the ischemic cortex is reduced in Aim2 knockout mice [21]. Finally our findings also confirm that ASC and caspase-1 (and IL-1β) levels are increased after OGD (or OGD/LPS) in both microglia and macrophages in accordance with published findings [21] [27] [28].

Conclusions

Herein we demonstrate that microglia/macrophages upregulate the expression of Aim2, ASC, caspase-1, NRLP3, and IL-1B after being exposed to an *in vitro* model of ischemia. We contend that a further elucidation of molecular and cellular mechanisms related to ischemic pathobiology may ultimately lead to advanced targets/novel therapeutics capable of significantly reducing morbidity/mortality following ischemic brain injury.

Limitations

We acknowledge that there are limitations within our study. Accordingly we feel it is prudent to highlight that recent evidence has emerged to suggest that cultured primary microglia may in fact be a poor model of microglia *in vivo* [29] [30] [31].

We will seek to identify key regulators of this response (i.e., those capable of upregulating Aim2, ASC, caspase-1, NRLP3) via ongoing genomic/*in silico* analyses. We hope

to add our pending results to this report thereby embracing the modular design of the journal and concordantly increasing the impact of our initial observation.

Additional Information

Methods

Reagents/kits

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CA; #AG-25B-0006), anti-AIM2 (Santa Cruz Biotechnology, Santa Cruz, CA; #SC137967). Signals were detected using a chemiluminescent substrate (Immobilon Western; Millipore, Billerica, MA) followed by digital imaging (Alpha Innotech, San Leandro, CA) or C-Digit (LI-COR, Lincoln, NE).

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Supplementary Material

Please see https://sciencematters.io/articles/201602000017.

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Ethics Statement

All animal procedures were reviewed and ultimately approved by the National Institute of Neurological Disorders and Stroke (NIH), Animal Care and Use Committee.

Citations

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