The Arginase Pathway in Neonatal Brain Hypoxia-Ischemia

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Abstract

Brain damage after hypoxia-ischemia (HI) occurs in an age-dependent manner. Neuroprotective strategies assumed to be effective in adults might have deleterious effects in the immature brain. In order to create effective therapies, the complex pathophysiology of HI in the developing brain requires exploring new mechanisms. Critical determinants of neuronal survival after HI are the extent of vascular dysfunction, inflammation, and oxidative stress, followed later by tissue repair. The key enzyme of these processes in the human body is arginase (ARG) that acts via the bioavailability of nitric oxide, and the synthesis of polyamines and proline. ARG is expressed throughout the brain in different cells. However, little is known about the effect of ARG in pathophysiological states of the brain, especially hypoxia-ischemia. Here, we summarize the role of ARG during neurodevelopment as well as in various brain pathologies.

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Introduction

Hypoxic-ischemic brain injury accounts for a significant proportion of mortality and long-term disability in children, affecting 0.7–1.2 million infants annually [1]. While progress in respiratory and intensive care technology has greatly improved survival rates, the incidence of motor and cognitive disorders linked to perinatal and early postnatal brain injury has actually increased in the last 2 decades [2]. Despite the significant socioeconomic burden of neonatal hypoxia-ischemia (HI), there are currently very few preventative and/or protective therapies available for patients who have suffered brain injury from HI, with only one treatment licensed for use, i.e., hypothermia. The etiology of HI is complex, with a clear initial primary-insult phase followed by a more delayed, secondary-injury phase that can continue for long periods of time. While the initial, primary insult contributes to neuronal cell death, the degree of secondary injury induced by inflammation and oxidative stress is perhaps the more critical determinant of neuronal survival. Many factors contribute to cell death in this extended injury phase [3] and, given its duration, this phase can easily be considered the most viable time period to initiate effective therapy.

Fig. 1. Schematic diagram of relationship between ARG and NOS isoforms in the brain. NOS, nitric oxide synthase; ARG, arginase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; NO, nitric oxide; GABA, γ -aminobutyric acid.

Arginases (ARG) are enzymes expressed throughout the brain in 2 known isoforms and have conventionally been studied in their role as the penultimate step in the urea cycle [4]. However, more recent evidence reveals the breadth of different roles that ARG play, particularly in various disease states, in both beneficial and detrimental ways [5]. Given that ARG are among the most acutely upregulated enzymes in models of ischemic injury (of the heart [6], liver [7], and retina [8]) and key regulatory enzymes of many inflammatory states, elucidating the molecular mechanisms of the ARG pathway will enable the development of more effective therapies to improve outcomes. We speculate that it is ultimately the complex interplay between the individual characteristics of the ARG isoenzymes, their interactions with other coregulators, and their temporal expression patterns that determine the specific effects of ARG, particularly in the central nervous system (CNS) after hypoxic-ischemic injury.

Arginase

ARG Isoforms and Expression

ARG is expressed as 2 isoforms, arginase 1 and 2 (ARG-1 and ARG-2), that differ in their genetic coding, subcellular localization, tissue distribution, immunological cross-reactivity, molecular regulation, and function [9, 10]. ARG-1 is a cytosolic, homotrimer enzyme with a 35-kDa subunit [9] encoded by the *arg1* gene located on

chromosome 6q23 [11]. It is constitutively and abundantly expressed in the liver, and some ARG-1 expression has also been reported in several extrahepatic tissues such as the brain, stomach, pancreas, and lung [10, 12]. The second isoform, ARG-2, is a 40-kDa, mitochondrial enzyme encoded by the *arg2* gene located on chromosome 14q24.1–24.3 [13]. Unlike ARG-1, ARG-2 is confined mainly to the kidney, brain, prostate, intestine, and pancreas [10, 12–14]. Both isoforms of ARG share a similar structure, with >50% homology of their amino acid residues, and 100% homology in the areas critical for their L-arginine metabolizing function [13–15]. This suggests an overlap in their metabolizing functions, but their individual functional impact is highly specific to cell and organ types.

ARG Functions

ARG is a metalloenzyme involved in the urea cycle, a series of biochemical reactions that produce urea from ammonia for excretion. Specifically, it is involved in the penultimate step, the hydrolysis of the semiessential amino acid L-arginine to form L-ornithine and urea [4]. The breakdown of L-arginine plays an important regulatory role in the formation of active biomolecules including nitric oxide (NO), citrulline, polyamines, and proline among others (Fig. 1).

The primary function of ARG-1 is detoxification by removing excessive nitrogen produced from amino acid metabolism through the hepatic urea cycle [4]. ARG-1 knockout mice exhibit severe symptoms of hyperammo-

nemia and do not survive past postnatal day 10 (P10)–P14 [16], while patients with genetic mutation-induced ARG-1 deficiency demonstrate hyperammonemia and symptoms that mostly involve the CNS, like progressive spastic paraparesis, epileptic seizures, and psychomotor and growth retardation [17]. On the other hand, ARG-1 overexpression and an increase in ARG activity have been associated with various complex disease states, implicating ARG as a key regulatory enzyme in inflammation [18] and repair [19]. Examples are: pulmonary disease [20], diabetes [21], carcinogenesis [22], hypoxic-ischemic and reperfusion injury in different organs [23–28], and cardiovascular disease [23, 29].

While the overall role of ARG-2 has remained unknown for some time, more recent studies have begun to uncover its role in vascular endothelial damage via oxidative stress [30–32] and mitochondrial dysfunction [33]. Identified as the predominant isozyme in human and mouse endothelial cells [34, 35], ARG-2 is implicated in the regulation of endothelial senescence [30, 35] and function in many disease states [34, 36, 37]. Specifically, ARG-2 hydrolyzes L-arginine in vascular endothelial cells, thereby limiting its availability for the generation of NO via endothelial NO synthase (eNOS), and resulting in vascular endothelial dysfunction [38], oxidative stress, and the enhanced expression of endothelial inflammatory adhesion molecules [30]. Loss of both genes for ARG-2 in ARG-2 knockout mice leads to a systemic hypertension phenotype with a blunted response to vasoconstriction [39]; this highlights a prominent role for ARG-2 in endothelial dysfunction.

The discovery of ARG-1 and ARG-2 expression in the brain has raised the question of whether a complete urea cycle occurs in brain and what its role might be. Previously, it was claimed that the complete urea cycle may not occur in the brain; however, many of the recent reports to have emerged from the study of Alzheimer's and Huntington disease established the presence of elevated urea levels and urea cycle genes in the brain [40, 41]. These findings have garnered much interest in the role of ARG in the brain, and studies have started focusing on the regulation of ARG and other elements in specific disease states and how modulating members of the urea cycle may affect these disease states. Data on the role of ARG after brain HI are limited, and some are conflicting. However, all recent studies point out that ARG responds to HI conditions in the brain and may play a pivotal role in neural injury, especially via the NO pathway and repair via the ARG effect on polyamine synthesis.

ARG and the NO Pathway

NO is a well-known signaling molecule of great interest, particularly in the CNS. It is produced by NOS, an enzyme with many cellular isoforms that regulate the expression and function of NO based on cellular location. Neuronal (n)NOS (NOS-1) and eNOS (NOS-3) are commonly associated with the nanomolar levels of NO production that mediate intracellular signaling processes and vascular homeostasis, respectively [42]. Inducible (i)NOS (NOS-2) produces high levels of NO in the micromolar range and plays an important role in tissue inflammation and host defenses [43]. The substrate for NOS activity and NO formation is L-arginine, and, consequently, NOS function is reciprocally regulated by ARG via substrate depletion [42]. Interestingly, although the affinity of L-arginine is for purified NOS (Km approx. 2–20 μ M) is much higher than for ARG (Km approx. 1–5 mM), as the maximum activity of ARG is >1,000 times that of NOS, similar rates of substrate utilization occur at physiologic L-arginine concentrations [15].

Several findings suggest that the limitation of NO production by ARG, and therefore its physiologic function, is critically dependent on the particular combination of ARG isoform with NOS isoform. Upregulation of either ARG-1 or ARG-2 is effective in limiting NO production by iNOS; however, considering that the rate of L-arginine utilized by nNOS is much lower than that used by iNOS, a similar outcome cannot be assumed for nNOS [42]. The decrease in nitrite production by nNOS correlates with an increase of cytosolic ARG-1 activity but not mitochondrial ARG-2 activity [42]. For eNOS, however, the overexpression of ARG-2 is able to inhibit NO production, although the effect is smaller than that of transgene ARG-1 [44], thus highlighting the specificity of enzyme function based on isoform and location. Interestingly, the intermediate in NO synthesis, N ω -hydroxy-L-arginine, and the ARG product L-ornithine, decrease ARG activity, suggesting there are multiple factors in the regulation of ARG-NOS interactions and metabolic pathways [20].

ARG and Polyamine and Proline Pathways

The effect of ARG on polyamine or proline synthesis is dependent on the subcellular colocalization of the ARG isoforms with the enzymes of polyamine or proline pathways. The colocalization of ARG-1 with ornithine decarboxylase in the cytosol directs ornithine, a product of ARG metabolism, towards polyamine synthesis [44]. In contrast, when ARG-2 is colocalized in mitochondria with ornithine aminotransferase, ornithine preferentially forms proline and glutamate [44]. Considering that orni-

thine is a precursor for the neurotransmitter synthesis of both glutamate and γ -aminobutyric acid (GABA) [45], the cellular localization and activities of these 2 isoforms could play a major role in mediating neurotransmitter levels and the subsequent responses to injury processes. However, in ARG-1^{-/-} mice, while there was a mild decrease in net brain glutamine compared to littermate controls, GABA levels remained unchanged [46], suggesting additional regulatory mechanisms and not ARG-1 alone. Polyamines (namely putrescine, spermidine, and spermine) are intracellular, and interact with nucleotides and phospholipids and, consequently, are involved in many functions related to cell survival, proliferation, maturation, and neurite growth among others [47, 48]. Polyamines can also exert harmful effects, such as neuronal damage from excitotoxicity via the overactivation of N-methyl-D-aspartate (NMDA) receptor [49]. Therefore, depending on the context, ARG may play a beneficial or detrimental role via the modulation of excitotoxicity or tissue repair.

The ARG Pathway in the Brain

ARG Localization in the Brain

Within the CNS, the 2 isoforms of ARG differ in their regional, cellular, and subcellular expression patterns. ARG-1 and ARG-2 are both expressed throughout the brain of chickens [50] and rodents [12], with ARG-1 expressed at higher levels than ARG-2 [12]. In rats, expression of ARG-1 and ARG-2 has been detected in the cortex, hippocampus, thalamus, basal ganglia, cerebellum, brainstem, and spinal cord among other substructures [10, 12, 51–53]. Within these regions, ARG has been noted to be localized to variable cellular subtypes. In the hippocampus, ARG expression is found in both excitatory and inhibitory neurons [51]; in the cerebellum, expression has been noted in basket, stellate, Golgi, and Purkinje cells [51, 52]. Although some initial studies suggested that ARG expression is confined to the neurons [12], further study clarified that both isoforms are expressed in the glia, i.e., oligodendrocytes and microglia [10, 51, 53], and in astrocytes [54]. It remains to be elucidated which cells represent the major sources of ARG in HI conditions of the brain, and what changes occur in terms of ARG activity and expression as the injury evolves.

ARG Spatiotemporal Changes in the Brain with Age

Damage in the neonatal brain after HI is both region- and cell-specific [3] and involves development-specific

processes that are markedly different from in the adult brain [55]. Therefore, understanding ARG spatiotemporal changes during development is critical.

ARG expression and activity both undergo spatiotemporal changes with age. ARG-1 expression in the murine brain and in the ganglion cells of the peripheral nervous system have been noticed as early as embryonic life; positive staining is seen from embryonic day (E)13 to P1, and peaks at E15–E17 [56]. Expression is evident in the cervical, thoracic, and lumbar dorsal-root ganglia (DRG), confined to sensory neuron cell bodies from E13 to P1, in the vagal nucleus, and in the medulla at E17 [56]. Relatively high expression of ARG-1 was found in microglial cells early in the postnatal period (P3), and this had diminished by 70% at P21 and by >90% at 12 months [57]. This pattern parallels that of microglial iNOS, suggesting a temporal link between the 2 enzymes that utilize L-arginine, though the details of these interactions in microglial development are yet to be elucidated [57]. ARG-2 expression, on the other hand, is undetectable at earlier stages (E13–P1) [56] but is evident in adulthood [12, 53]. Similarly, ARG expression peaks in the brain and retina shortly after the chicks hatch, and then slowly declines by adulthood [50].

ARG activity appears to change in association with changes in brain molecular structure and function. In rats, gross ARG brain activity is highest in fetal and neonatal brains, and then decreases 4-fold by adulthood [58]. In young adult rats, ARG activity is highest in the postrhinal cortex and in subregions of the hippocampus, particularly the dentate gyrus, followed by CA2/3, and then CA 1. Lower ARG activity has been found in the temporal cortex, and the lowest is in the entorhinal and perirhinal cortices [59, 60]. There are no significant differences in ARG activity or expression in the prefrontal cortex in young and aged rats [61]; however, ARG activity in the postrhinal cortex decreases with age [59, 60]. These findings conflict somewhat with another study by the same group that found a significant increase in ARG activity only in the perirhinal cortex of aged rats [62], and a similar finding was described in an animal model that compared 1-month-old and 14-month-old mice [63].

ARG activity does not, however, appear to correlate with expression levels, as the highest expression of ARG-1 has been noted in postrhinal and perirhinal cortices and significantly lower expression in the entorhinal cortex, dorsal hippocampus, and temporal cortex [60]. ARG-2 expression, in contrast, does not vary by region. As the mice aged, no change was noted in the expression of either isoform, ARG-1 or ARG-2, across hippocampal re-

gions. There was a clear temporal pattern for hippocampal ARG activity, however, which decreased with age in CA1 and CA2/3, but not in the dentate gyrus.

These studies draw attention to the fact that spatio-temporal and age-dependent variations in ARG expression and activity clearly exist in the brain and are perhaps indicative of the differential regulation of each of these regions during development and, conceivably, after injury.

The Role of ARG in Neurodevelopment

While several studies have now established the importance of ARG and its downstream products in embryogenesis and placental development, few have specifically investigated the role of ARG in early neurodevelopment.

ARG activity is noted to be highest during the periods of highest protein and polyamine synthesis [58, 64, 65], suggesting a pivotal role for this enzyme that starts early in development. ARG knockout animals exhibit decreased dendritic complexity, intrinsic excitability, synapse numbers, and functional synaptic deficits, consistent with the anatomical changes observed with an unexpected gradation of abnormalities based on whether it is a single- or double-copy loss of *arg1*. Gene therapy with *arg1* at neonatal stages rescues nearly all of these abnormalities [66].

Intriguingly, however, neural stem cells isolated from the germinal zones of ARG-1 knockout embryos were found to be capable of differentiating into neurons, oligodendrocytes, and astrocytes [67]. They appeared to mature more rapidly than wild-type and heterozygous stem cells, elaborating for longer, with more complex neurites, and more frequently expressing mature neuronal markers such as β 3-tubulin and neurofilament. It is possible that a compensatory upregulation of agmatinase and spermine synthase in ARG-1 knockout neural stem cells may upregulate alternative polyamine synthesis pathways and intermediate substrates, circumventing the loss of ARG-1 to continue polyamine-dependent proliferation [67]. Together, it appears that while alternative pathways of polyamine synthesis may enable the anatomical differentiation and maturation of neurons in states of ARG deficiency, ARG is still necessary for the formation of the dendritic network and synaptic connections.

In early neurons, the strict temporal control of neuronal ARG expression appears to have developmental consequences for axon growth. In cultured DRG neurons [47], ARG levels were initially high in the young neurons; this correlated with the myelin-induced promotion of axon growth early in development. However, with a pre-

dictably timed drop in ARG activity at P4, the effect of myelin rapidly switched to inhibit axon growth. It was suggested that neurotrophin signaling elevated cAMP levels, activating the transcription of ARG-1 which then indirectly increased levels of polyamines, leading to axon growth by overcoming the inhibitory signals of myelin. When cAMP spontaneously dropped at age P4, this molecular pathway was downregulated, and inhibitory signaling from myelin prevented further axon elaboration.

ARG-1 is thus thought to play a fundamental role in the promotion of axon growth, by improving the ability of axons to overcome the actions of inhibitory factors such as myelin-associated glycoprotein (MAG) in late postnatal neuronal development and also after CNS injury. Overexpression of ARG-1 and the addition of polyamines both enhanced axon growth in cultured cerebellar neurons grown on myelin, while inhibitors of ARG and ornithine decarboxylase blocked axon growth, presumably by preventing the conversion of arginine to ornithine or ornithine to polyamines, respectively [47].

ARG clearly plays a critical role in neurodevelopment, neurogenesis, and axonal repair mechanisms. As such, understanding the developmental patterns and modulation of this pathway following injury, especially after HI, is a critical first step towards developing effective therapeutic strategies.

ARG Response after Brain Injury

Changes in ARG Expression and Activity

To date, 3 phases of injury progression have been described after neonatal brain HI: the acute primary energy failure due to the hypoxic-ischemic insult; a secondary, subacute phase which is a consequence of reoxygenation and reperfusion; and a tertiary, chronic phase in which previous events can get worse and inflammation becomes chronic [68]. ARG appears to respond to all phases by changes in its activity and expression.

In a rat model of anoxia-hypoxia, ARG expression remained unchanged during periods of anoxia but increased significantly during the reperfusion phase 5 days after the insult [69]. Similarly, Quirié et al. [70], using a rat photothrombotic model of brain ischemia, observed that while the ARG-2 isoform was not modified by the injury, ARG-1 expression increased at the injury site on day 8, peaking on day 15. They also noted a 29% decrease in ARG-1 activity compared to controls, postulated to be due to a possible delayed expression of endogenous ARG inhibitors. Another report, by Hamzei Taj et al. [71],

Table 1. Summary of the studies describing the ARG pathway after stroke, hypoxia, and traumatic brain injury

First author [ref.], year	Animal model	Timeline for measurement of changes in ARG pathway after injury	Adult/pup	Observed effect(s)
Ahmad [24], 2016	Male C57BL/6 wild-type and Arg-2 knockout mice; MCAO	day 7	adult	Higher infarction volumes and neurologic deficits; excitotoxic damage
Hamzei Taj [71], 2016	Male C57BL/6 wild-type mice; MCAO	days 6 and 14	adult	ARG-1 expression highest 1st week; number of Arg-1+ microglia/macrophages correlated with neuronal protection and functional improvement after miRNA-124 treatment
Barakat [141], 2018	Male Wistar wild-type rats; MCAO	not stated	adult	Increased ARG-1 and ARG-2 expression inhibited by L-citrulline and L-ornithine treatment; reduction of infarct size and brain edema
Clarkson [25], 2004	Male Wistar wild-type rat pups; MCAO	day 7	pup	Significant decrease in infarction size and ARG activity after spermine supplementation
Clarkson [26], 2005	Male Wistar wild-type rat pups; MCAO followed by hypoxia	days 3 and 90	pup	Increased ARG activity after HI on day 3, persistent increase on day 90 after HI; decreased ARG activity after clomethiazole; neuroprotection
Gao [135], 2017	Male C57BL/6 wild-type mice; TBI: CCI	day 3	adult	Treatment with IL-2C significantly increased the number of ARG-1+ cells around the lesion area
Xu [136], 2017	C57BL/6 wild-type mice; TBI model: CCI	days 1 and 3	adult	Increased ARG-1 expression in M2 microglia after atorvastatin therapy
Bitner [142], 2010	C57Bl6/J and ARG-2 knockout mice; TBI: CCI	15 min and 1 h	adult	The absence of ARG-2 significantly improved CBF recovery after trauma
Swamy [69], 2010	Male Sprague-Dawley wild-type rats exposed to anoxia for 4–5 min	day 5	adult	Increased ARG expression; activity increased in reperfusion phase
Quiríe [70], 2013	Wistar wild-type rats; photothrombotic stroke model	days 1, 8, 15, and 30	adult	Increased ARG-1 expression on day 8; ARG-2 unchanged; activity highest on day 8

HI, hypoxia-ischemia; MCAO, middle cerebral-artery occlusion; TBI, traumatic brain injury; CCI, controlled cortical impact; CBF, cerebral blood flow.

showed ARG-1 expression to be strongest within the first week after middle cerebral-artery occlusion (MCAO), and decreasing by the second week. Another study observed that a higher ARG-1 activity persists on day 90 after injury, suggesting that ARG-1 remains elevated for a longer period of time [26].

In terms of spatiotemporal expression after ischemic insult, increased ARG-1 expression localizes to activated microglia within the lesion core, whereas the neuronal ARG-1 expression is strongest in cortical neurons located in the nonaffected hemisphere. Lower ARG-1 expression is also found in astrocytes sporadically located within the glial scar [71]. In a photothrombotic model of ischemia, ARG-1 was found to be higher in activated macrophages, neurons, and astrocytes in the area of the lesion [70]. Interestingly, ARG-1 expression in astrocytes follows a similar pattern to that of brain derived-neurotrophic factor (BDNF), indicating a possible role of ARG in BDNF regulation, neuronal survival, growth, and neuroplasticity [70, 72].

It appears that the observed differences in ARG localization, expression, and activity in response to brain HI are age- and animal model-dependent, making it vital to extrapolate findings in a similarly appropriate manner to

ensure accurate assessment (Table 1). Understanding the dynamics of ARG in response to brain hypoxic-ischemic insult and the role played by ARG in the pathophysiology of the injury at particular time points has important implications for appropriate therapeutic targeting of ARG pathway. In the immediate phase of injury, it may be the influence of ARG on the NO pathway that plays a significant role. While the effects of ARG inhibition might be detrimental, by increasing the availability of L-arginine for iNOS and nNOS as previously stated, it is possible that it is also beneficial by improving cerebral perfusion and attenuating vascular oxidative stress via the eNOS pathway. We postulate that inhibiting ARG in the acute phase of brain HI may decrease neurotoxicity by decreasing polyamine synthesis. However, ARG inhibition might not be favorable during the later stages of injury since polyamines and proline participate in tissue repair.

ARG Regulation in the HI Environment

HI creates a molecular environment of vascular dysregulation, oxidative stress, inflammation, and excitotoxicity that lead to progressive damage over a long period of time [3]. This damage is a critical determinant of permanent neuronal loss and poor clinical neuro-

logical outcomes. Various mechanisms activated in response to neonatal brain HI alter the ARG metabolic pathway.

Hypoxia

The hypoxic environment induces both ARG expression and activity [73]. ARG-2 is stimulated by HIF-2 α [74], and HIF-2 α is one of the predominant adaptive responders to acute brain hypoxia in the oxygen-deprived environment after stroke [75]. Hypoxic upregulation of ARG-2 expression has been shown to also occur via AMPK α 1 signaling [76], miRNAs [77], the PI3K-Akt pathway [78], the direct induction of the ARG-2 promoter over ARG-1 [79], and the activation of the extracellular signal-regulated kinase or epidermal growth factor receptor tyrosine kinase, which stimulate both ARG-1 mRNA and ARG2 mRNA expression [80–82]. Interestingly however, ARG-2 mRNA is suppressed by >50% by O₂ deprivation [83], suggesting that the exact influence of hypoxia on ARG in the brain remains to be clarified further.

Ischemia and Reperfusion Injury

ARG is upregulated during ischemia-reperfusion, and whether this is protective or detrimental is organ-dependent. In models of myocardial infarction, ARG-1 is one of the most abundant and fastest-upregulated genes [6, 84] with increased activity [85–87] following ischemia-reperfusion injury. This upregulation has been shown to be largely detrimental, mostly likely by decreasing NO levels [87] and bioavailability [88]. The transcription factors Forkhead box O4 (FoxO4) [89] and tumor necrosis factor α (TNF- α) [90] have both been implicated in mediating this upregulation. In retinal ischemia-reperfusion injury models, ARG-2 deletion leads to significantly reduced glial activation, reactive oxygen species formation, and cell death by necroptosis, leading to decreased ganglion cell loss, microvascular degeneration, and preserved retinal morphology [8]. Similarly, in the small intestine [91] and liver [92], ARG blockade has been shown to increase L-arginine bioavailability, resulting in less injury in the liver following ischemia-reperfusion. Essentially, in reperfusion models of organs other than the brain, ARG upregulation following injury appears to have detrimental effects. While data from the brain are limited, a recent study has shown that deletion of ARG-2 in cerebral ischemia has detrimental effects, with higher infarction volumes, excitotoxic damage, decreased cerebral blood flow, and higher neurologic deficit scores [24], which clearly highlight organ-specific roles for this enzyme and its isoforms.

ARG and the Molecular Mechanisms of Brain HI

The source of ARG in tissue after hypoxic-ischemic injury varies. ARG may be released from damaged tissue cells, newly expressed in cells at the injury site, or originate from cells that migrate into the lesion like macrophages or microglia. In a model of spinal cord injury and autoimmune encephalitis, ARG-1 was expressed exclusively in infiltrating myeloid cells and not in microglia [93]. Zarruk et al. [94] observed a similar pattern, where macrophages upregulated the expression of ARG-1 mRNA and ARG protein as a response to brain ischemia more than microglia did. The definitive sources of ARG after HI remain to be established. Different types of brain injury must be taken into consideration. For example, brain cells exposed to hypoxic injury may have an expression profiles that is different from in cells exposed to both hypoxia and ischemia.

ARG and Neuroinflammation

Neuroinflammation is a critical aspect of injury and postinjury mechanisms in the brain, and it is conceivable that the regulators of neuroinflammation may play an important role in mediating both injury and repair processes. Many factors involved in neuroinflammation have been shown to regulate ARG, which highlights the pivotal role for ARG in such processes.

Selected inflammatory cytokines vary in their ability to stimulate ARG. Interleukin (IL)-13 and IL-4 induce ARG-1 overexpression via cAMP and the activation of JAK/STAT6 pathways [95, 96], while IL-13 induces expression of ARG-2 by its STAT3- but not its STAT6-mediated effect on IL-13R α 2 [97]. IL-10, on the other hand, stimulates ARG-1 minimally [98]. While the stimulatory effects of ILs have been well established, data on the effects of interferon (IFN)- γ are inconclusive. Multiple studies have shown that IFN- γ inhibits the activity of both ARGs [99], ARG-1 [98] and ARG-2 [100], although at least 1 report is contradictory [101]. Interestingly, in a murine model of autoimmune encephalitis, ARG inhibition with 2(S)-amino-6-boronohexanoic acid (ABH) led to decreased production of IFN- γ [102].

The regulation of ARG by cytokines appears to be a more complex process, where suppressors of cytokine signaling (SOCs) play a role via negative feedback inhibition on the JAK/STAT signaling pathway [103]. SOCs1 and SOCs3 are both expressed in microglia [103], and they regulate microglia activation [104] and macrophage polarization. Interestingly, this regulation is associated with changes in ARG/iNOS ratio; SOCs1 leads to a high

ARG-to-iNOS ratio and M2 activation [105]. Knockdown of SOCs1, on the other hand, decreases M2-induced ARG-1 expression and activity, but also reciprocally enhances iNOS activity, SOCs3 expression associated with M1 activation [105], and the suppression of ARG-1 activity [106]. Considering that microglia are one of the first responders to injury in the neonatal brain and have been implicated in both beneficial and detrimental effects, these findings are critical to the understanding of postinjury processes.

Prostaglandin (PG)E1, PGE2, PGE3 [107], and transforming growth factor (TGF)- β 1 [108] have also been shown to modulate ARG expression.

ARG and Excitotoxicity

ARG2^{-/-} knockout mice have been shown to have increased injury and neurologic impairment caused by MCAO and excitotoxic injury, likely due to L-arginine being redirected to the excitotoxicity-induced NOS pathway which leads to downstream oxidative stress [24]. Interestingly, however, it appears that polyamines, produced by the ARG metabolite ornithine, may exacerbate excitotoxicity by directly binding NMDA receptors [49]. Intraocular NMDA injections upregulate ARG-1, which presumably leads to increased ornithine and polyamine production, pharmacologic blockade of polyamine synthesis, and competitive blockade of polyamine binding sites on NMDA receptors, reducing excitotoxic retinal ganglion cell death [49].

A significant component of neuronal injury after neonatal brain HI results from excitotoxicity that stems from the pathologic release of excess glutamate. As studies have begun to elaborate the downstream signaling pathways that lead from glutamate receptor binding to cell death, most of the damage is thought to result from the oxidative stress induced by NOS upregulation and the increased production of NO. As NOS and ARG share the substrate L-arginine, many models seem to suggest that the shunting of L-arginine by the NOS pathway is neurotoxic, while alterations that would lead to increased ARG utilization of L-arginine might be neuroprotective. Competition between these 2 pathways may require a more delicate balance, however, as the polyamines that are produced downstream of ARG metabolites may themselves be neurotoxic [54]. Existing studies have just begun to aid our understanding of ARG metabolism in excitotoxic injury, but more study will be necessary to understand exactly how to harness these enzymatic pathways to maximize neuroprotection and minimize neuronal death.

ARG and Apoptosis

It has been found that depletion of L-arginine by ARG results in neuronal starvation and impaired neuronal survival, inducing apoptosis and subsequent neuronal death [109]. In the same study, inhibition of ARG-1 with difluoromethylornithine (DFMO) reversed abnormal L-arginine utilization, suggesting a causal link between ARG activity and apoptotic death. In conflict with this report, however, overexpression of ARG-1 was found to prevent neuronal death, potentially via the suppression of NO production, and subsequent peroxynitrate production combined with an increase in polyamine synthesis [47]. ARG has also been shown to promote cell survival via the intracellular depletion of L-arginine, leading to the activation of the serine/threonine kinase, GCN2, to phosphorylate translational initiation factor 2 α (IF-2 α) associated with cell survival [110, 111].

ARG and Oxidative Stress

The neonatal brain is selectively vulnerable to oxidative stress [112]. ARG and oxidative stress have a reciprocal relationship. ARG activity is stimulated by free radicals and activated ARG decreases the bioavailability of the NOS substrate, L-arginine, further increasing oxidative stress [113–115]. Hydrogen peroxide (H₂O₂) and peroxynitrite (ONOO⁻) lead to increased ARG activity and expression via protein kinase C activation and subsequent RhoA/Rho-associated kinase activation [115], and this mechanism likely also occurs in microglial cells after injury [116].

In addition to this, ARG contributes to depletion of the substrate L-arginine for all 3 NOS isoforms, and NOS uncoupled from its substrate generates superoxide and/or H₂O₂, a feature characteristic for all NOS isoforms [117]. The specific downstream effects of substrate depletion may vary depending on the NOS isoform; while eNOS uncoupling leads to radical formation resulting in endothelial damage, the deprivation of L-arginine from iNOS is associated with a neuroprotective role of ARG, given the observed improvement in oxidative and nitrosative stress. The effects of substrate depletion may also differ with timing after injury. For example, during reperfusion, uncoupled iNOS is a source of free radicals and can contribute to significant reperfusion injury [117].

The contribution of L-arginine availability for NOS to radical formation is NOS isoform-dependent. While increased L-arginine availability for nNOS decreases the rate and amount of total radical production, in eNOS, it is only the rate that is affected [118]. Besides impaired NO formation, ARG also decreases the pro-

duction of L-citrulline, which has been shown to have antioxidative properties [119], improve neuronal survival, and attenuate eNOS and nNOS expression after ischemic injury [120], restore NO levels, increase eNOS, and suppress iNOS after glutamate toxicity [121]. Treatment with L-arginine, on the other hand, increases iNOS [121]. All these findings indicate very fine-tuned roles for ARG in mediating oxidative stress and downstream injury mechanisms, thus highlighting the need for careful consideration of its effects, specifically on the NOS pathway.

ARG and the Regulation of the Blood-Brain Barrier

The disruption of the blood-brain barrier (BBB), impaired cerebral autoregulation, and local changes in perfusion are the vascular pathophysiological features of HI and reperfusion injury of the brain. The role of ARG in vascular regulation and endothelial senescence has been extensively studied in several peripheral organs, and it is likely that at least some of these effects could be extrapolated to the brain. As ARG-1 and ARG-2 have both been detected in the cerebral microvasculature, it is assumed that ARG affects cerebral microcirculation, BBB integrity, and endothelial function (review [31]). The primary isoform in endothelial cells is ARG-2 [38], which, as discussed earlier, interacts with eNOS to regulate the production of NO [122]. Decreasing L-arginine availability for eNOS by ARG leads to eNOS uncoupling [123], which promotes oxidative stress. Upregulation of ARG leads to the induction of an endothelial senescence phenotype, eNOS-uncoupling, elevated adhesion molecule expression, and enhanced monocyte-endothelial cell interaction. While ARG-2 exhibits these effects through S6K1-signaling [30], ARG-1 does not [32]. Multiple vascular factors act as ARG inducers at the site of vascular injury. For example, the release of TGF- β [124], or thrombin in endothelial cells which modulate endothelial ARG gene expression via the AP-1 promoter sequence-bound transcription factors c-Jun and ATF-2 [125]. Besides effects on local microcirculation, ARG stimulated by brain injury may also impact the systemic vasculature. ARG-1 expression increased in mesenteric arteries in a rat model of traumatic brain injury (TBI), and supplementation with L-arginine or ARG inhibition with nor-NOHA reversed the ARG effects [126]. However, the clinical significance of systemic changes in ARG expression after brain injury is unknown.

Clinical Applications

ARG as a Biomarker

Both gene expression and serum activity of ARG-1 have been shown to increase in adult patients with acute ischemic stroke, and, more importantly, correlate with infarct volume and the severity of the stroke [127], suggesting a considerable role for ARG as a biomarker after stroke. Interestingly, ARG gene expression and serum protein levels also correlate with increases in peripheral neutrophil counts [127] which has been associated with poor neurological outcomes in asphyxiated newborns [128]. The relationship between ARG expression and neutrophils might be different in newborns, however, since neutrophils show different patterns of recruitment after stroke and migrate to the injury site at later time points than in adults [129]. The utility of ARG as a biomarker is possible but needs to be examined further in validation cohorts.

ARG as a Therapy

Given all the evidence thus far, the potential role of multiple steps of the ARG pathway in brain injury may represent a promising therapeutic target of neonatal brain HI. Manipulation of the ARG pathway at certain time points after the hypoxic insult may provide neuroprotection and potentially also enhance neurorestoration. To do this, however, besides establishing the appropriate time window for ARG inhibition, it is also important to characterize the effects of inhibition of the specific isoform of this enzyme. For example, in macrophage subpopulations, selective inhibition of ARG-1 in profibrotic M2 macrophages might lead to overexpression of the M1 inflammatory phenotype, which conversely predominantly expresses the ARG-2 isoform, and, in turn, aggravates iNOS-mediated inflammatory effects. On the other hand, inhibition of ARG-2 may enhance the profibrotic/repair M2 phenotype, with potential deleterious effects on vessels and other organs [130].

Different synthetic inhibitors of ARG have been produced, including boronic acid analogs, N-hydroxide-L-arginine, N-hydroxy-nor-L-arginine, and the newer (R)-2-amino-6-borono-2-(2(piperidin-1yl)ethyl) hexanoic acid [130]. Currently, although there are approximately 27 ARG inhibitors patented and studied [131], studies have highlighted the lack of specificity in most of these inhibitors. While some insight into the effects of ARG inhibition might come from knockout models, the results need to be evaluated carefully, owing to unknown compensatory pathways that might mask the ARG effects. Of course, the lim-

ited life-span of ARG-1 knockout also presents a considerable disadvantage. Despite this, however, ARG inhibitors have already been the subject of clinical trials showing promising effects in patients with cardiovascular disease (coronary artery disease, pulmonary hypertension, atherosclerosis, heart failure, vascular dysfunction in diabetes mellitus, and hypertension) [132–134].

The role of ARG inhibition after brain HI is unclear. While there are several studies describing the benefits of inhibiting ARG, some of these (discussed above) report the role of ARG as a neuroprotective enzyme. Comparing the effects of ARG inhibition is limited by the number of studies which are focused on different brain pathologies and the variety of time points for intervention.

The Benefits of ARG Activation

Increasing ARG activation/expression seems to be effective largely via the regulation of microglial polarization. Early treatment with miRNA-124, small noncoding RNA molecules involved in the posttranscriptional regulation of gene expression, has been shown to increase ARG-1 expression in immune cells, with polarization of macrophages/microglia towards the anti-inflammatory M2 phenotype associated with increased neuronal survival [71]. In mice subjected to TBI, administration of IL-2C increased the expression of *arg1*, among other genes, in the anti-inflammatory M2 microglia [135]. A similar effect has been observed in treatment with atorvastatin that increases M2 polarization and associated ARG-1 expression [136]. Considering that M2 microglia have been shown to elicit beneficial effects in the injured brain [137], these findings suggest modulating ARG expression after HI could have beneficial effects especially via microglial polarization. ARG catalytic function may be supported by manganese administration as shown in studies with a mouse model of Huntington disease, where manganese supplementation increased ARG-2 activity [138].

The Benefits of ARG Inhibition

In contrast, many studies have shown ARG inhibition following injury to be beneficial. In the brain, treatment with clomethiazole, associated with a decrease in ARG activity, was shown to be neuroprotective [139]. ARG inhibition has also been shown to decrease disease symptoms and accelerate recovery in a mouse model of autoimmune encephalitis [102], and to reduce injury volume in TBI [140] and MCAO [141], while ARG deletion significantly improves cerebral blood flow after TBI [142]. Caffeine is a commonly used medication with a high safety profile that decreases ARG activity; it acts via adenosine, a competitive

ARG inhibitor [143, 144]. As discussed earlier, it is likely that ARG has very specific effects based on age, timing, the organ affected, and isoform, and the conflicting nature of findings relating to ARG-mediated neuroprotection likely indicate the importance of careful extrapolation.

Conclusion

The ARG pathway is a complex metabolic pathway that is affected by multiple factors, and it appears that the ultimate effects do not occur in isolation, but rather as the culmination of complex interactions that can be species-, organ system-, and cell-specific. Currently, there are no specific inhibitors for the study of the effects on individual isoforms, and a knockout mouse shows a decreased life-span. Gender differences and gene polymorphisms may play a role. Despite these disadvantages, a better understanding of the effects of ARG on hypoxic-ischemic brain injury in newborns presents an attractive opportunity to develop new neuroprotective therapies.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Jana Krystofova was the principal author, wrote the manuscript, checked references, and gave final approval. Jeffrey Russ, Praneeti Pathipati, and Ann Sheldon wrote the manuscript, checked references, and gave final approval. Donna Ferriero supplied supervision, wrote the manuscript, checked references, final approval.

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