Ceftriaxone Preserves Glutamate Transporters and Prevents Intermittent Hypoxia-Induced Vulnerability to Brain Excitotoxic Injury

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Abstract

Hypoxia alters cellular metabolism and although the effects of sustained hypoxia (SH) have been extensively studied, less is known about chronic intermittent hypoxia (IH), commonly associated with cardiovascular morbidity and stroke. We hypothesize that impaired glutamate homeostasis after chronic IH may underlie vulnerability to stroke-induced excitotoxicity. P16 organotypic hippocampal slices, cultured for 7 days were exposed for 7 days to IH (alternating 2 min 5% O₂ - 15 min 21% O₂), SH (5% O₂) or RA (21% O₂), then 3 glutamate challenges. The first and last exposures were intended as a metabolic stimulus (200 μM glutamate, 15 min); the second emulated excitotoxicity (10 mM glutamate, 10 min). GFAP, MAP2, and EAAT1, EAAT2 glutamate transporters expression were assessed after exposure to each hypoxic protocol. Additionally, cell viability was determined at baseline and after each glutamate challenge, in presence or absence of ceftriaxone which increases glutamate transporter expression. GFAP and MAP2 decreased after 7 days IH and SH. Long-term IH but not SH decreased EAAT1 and EAAT2. Excitotoxic glutamate challenge decreased cell viability and the following 200 μM exposure further increased cell death, particularly in IH-exposed slices. Ceftriaxone prevented glutamate transporter decrease and improved cell viability after IH and excitotoxicity. We conclude that IH is more detrimental to cell survival and glutamate homeostasis than SH. These findings suggest that impaired regulation of extracellular glutamate levels is implicated in the increased brain susceptibility to excitotoxic insult after long-term IH.

Introduction

Restricted oxygen delivery alters brain cellular metabolism and increases astrocyte glucose uptake and lactate release to maintain viable energy levels and neuronal survival [1,2]. Although the effects of sustained hypoxia (SH) have been extensively studied, less is known about chronic intermittent hypoxia (IH) that has been shown to increase cardiovascular risks, and is commonly seen in diseases such as obstructive sleep apnea (OSA).

OSA has been identified as an important risk factor for stroke, independent of other risks such as hypertension, increasing the outcome severity and functional consequences [3,4,5,6]. Increased susceptibility to stroke in OSA patients has been mainly attributed to hypoxia-induced hemodynamic changes, as obstructive respiratory events elicit sympathetic and parasympathetic activation [6,7,8], that can be reversed by continuous positive airway pressure (CPAP) [9]. However, CPAP has not been unequivocally shown to decrease stroke susceptibility or improve stroke outcome [6]. Therefore additional cellular mechanisms may be induced by IH and play a role in OSA patients’ vulnerability to ischemic injury.

The role of excitotoxicity in the pathogenesis of ischemic brain disease has been widely reported [10] and elevated glutamate levels have been found in plasma and in cerebrospinal fluid of stroke patients [11]. Acute ischemia, as occurs during stroke, induces the release of glutamate and down-regulates glutamate transporter expression, critical to the regulation of intrasynaptic glutamate [11,12,13]. However, the effect of long-term milder sustained or intermittent hypoxia on these transporters is unknown.

Astrocytes maintain extracellular potassium/glutamate homeostasis and synaptic function during stroke [14,15]. In addition, astrocytes are critical for recovery after stroke and excitotoxicity and supply energy substrates for synaptic recovery following hypoxic injury [14,16]. Glutamate transporters, mainly GLAST (EAAT1) and GLT1 (EAAT2), play a critical role in the process [17,18]. EAAT2, primarily expressed in astrocytes, has been shown to be responsible for up to 90% of all glutamate uptake activity in the brain [19]. EAAT3, expressed mainly in neurons at very low levels, has been shown to play a role in neuronal metabolism rather than in glutamate transport [20]. The β-lactam antibiotic, ceftriaxone, increases GLT1 expression and function in rodents as well as in primary human fetal astrocytes and is neuroprotective in vivo in models of acute ischemia [21,22]. Therefore, increasing glutamate transporter expression during chronic exposure to hypoxia may decrease brain susceptibility to hypoxia and to excitotoxic injury.
These processes are difficult to study in commonly used preparations: cell culture models exclude cellular interactions, while animal models do not allow identification and modulation of cellular and molecular mechanisms. Incomplete ischemia is a common consequence of cerebral artery occlusion and glutamate uptake is critical to neuronal recovery after ischemia thus is a limiting factor in glutamate toxicity [14]. To determine whether glutamate excitotoxicity and glutamate transport play a role in OSA patient’s vulnerability to stroke, we exposed organotypic slices [23,24] to long term IH and SH. To evaluate the effect of IH and SH on slices survival to excitotoxicity, we determined slices baseline response to a low glutamate challenge after 7 days exposure to SH and IH, then to a high glutamate concentration, as may occur in a stroke. To assess whether IH or SH exposure affected slices’ ability to recover from excitotoxic challenge, high glutamate was washed away and a low glutamate concentration, similar to the first challenge, was applied. Our findings suggest that long term exposure to IH but not to SH alter glial glutamate transporter expression and compromises cell viability and brain response to excitotoxic insult that is critical during stroke. The beneficial effect of ceftriaxone treatment indicates that impaired glutamate transport plays a critical role in IH-induced vulnerability to ischemic brain injury.

Materials and Methods

Ethics Statement

Organotypic hippocampal slice cultures were prepared from 16-day-old Sprague-Dawley rats (Charles Rivers) as described in Stoppini et al., 1991 [23]. Rats were anaesthetized by intraperitoneal Nembutal injection, decapitated as approved by the University of Louisville Institutional Animal Use and Care Committee (protocol # 10100), and in agreement with the NIH guide for the care and use of laboratory animals.

Organotypic slice culture and experimental protocol

400 µm dorsal hippocampal slices were cut in ice cold dissection medium (HBSS with 25 mM HEPES and 6% glucose), using a McIlwain tissue chopper. Slices were cultured onto inserts (Millicell-CM, Millipore, Billerica MA), with growth media (50% MEM (Invitrogen, Carlsbad, CA), 25% horse serum, 25% HBSS, 20 mM HEPES, 1 mM glutamine, and 5 mg/ml glucose) in a controlled incubator chamber (Biospherix, Redfield, NY), as CO2, balanced N2) hypoxia, using a custom designed computer intermittent (IH; alternating 20 min 5% O2,- 10 min RA, 5% O2) hypoxia. Slices were collected previously performed in our laboratory [25]. Slices were exposed to RA, IH exposure for histological study, or incubated immediately after exposure for histological study, or incubated with propidium iodide (PI). Baseline images were captured after count and averaged for each experimental condition.

Statistics

SPSS IBM, v19 statistical software was used for data analysis. For viability studies, cell count data averaged over all experiments, averaging 4 fields per slice were analyzed using One-way ANOVA, and compared at baseline, at first 200 μM and at second 200 μM glutamate exposure. Differences among the various glutamate concentrations were compared within each of the three hypoxia conditions for Ceftriaxone-treated or untreated slices, using One-way ANOVA, followed by Tukey HSD post hoc tests. Immunofluorescence quantification was analyzed using one-way ANOVA followed by Tukey HSD multiple comparison tests.
Results

1. Effect of hypoxia on cell viability and susceptibility to glutamate

Cell viability was assessed, to determine the effect of long term exposure to IH and SH compared to RA. While in a cell culture model it would be relatively simple to quantify the total number of cells, cell density and slice architecture does not allow exact quantification in our organotypic slice model. Therefore, we have counterstained PI stainings after the various hypoxic exposures, using green hydrolyzed FDA staining. Living cells actively convert the non-fluorescent FDA into the green fluorescent fluorescein. Figure 1A shows decreased green fluorescence intensity after SH exposure, further decreasing after IH. In contrast, PI staining that stains damaged cells nuclei in red, increased in SH further increasing in IH. Confirming the PI data shown in Figure 1A, LDH release showed similar changes in viability, significantly increasing after SH and IH (Figure 2). To assess the effect of long

Figure 1. Long term sustained or Intermittent hypoxia decreases cell viability. Propidium iodide (red) and Fluorescein diacetate (green) staining of slices exposed to 7 days RA, SH or IH (A) without and (B) with 100 µM ceftriaxone (n = 4-6).
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term IH and SH on slice response to excitotoxicity, we determined slices baseline response to a non-excitotoxic glutamate challenge after 7 days exposure to RA, SH and IH, then to a high glutamate (10 mM) concentration, as may occur in a stroke. To assess whether slices exposed to IH or SH can recover from excitotoxic challenge, a low glutamate concentration, similar to the first challenge was then applied. Based on previous experimental evidence, the first and last glutamate exposures (200 μM glutamate for 15 min) should elicit a metabolic increase rather than excitotoxicity, as previously described in cultured astrocytes [26,27]; the second glutamate exposure (10 mM glutamate for 10 min) emulates excitotoxic insult, as 10-20 mM glutamate have previously been shown in our laboratory to induce excitotoxicity in slices [28], hence 10 mM was selected as a concentration that elicited moderate but not extensive excitotoxic cell death, as could occur in a transient ischemic event. Cell death assessed by PI was significantly higher at baseline, confirming the results showed in Figure 1A, and after each glutamate challenge in slices exposed to IH or SH, compared to RA (Figure 3A, 3B). As expected, the first 200 μM glutamate challenge that was intended as a metabolic stimulus [26,27], did not significantly affect viability in any of the conditions compared to their respective baseline. In contrast, excitotoxicity (10 mM glutamate) significantly increased cell death in all three conditions, with significantly higher toxicity in IH. Finally, the last 200 μM glutamate challenge significantly enhanced cell death in all three experimental conditions relative to baseline, and while the trend of higher IH toxicity was still apparent, there were no significant differences between conditions at this stage (Figure 3A, 3B).

2. Effect of ceftriaxone on cell viability and susceptibility to glutamate

Ceftriaxone has been shown to be neuroprotective in various models of ischemia, by upregulating glutamate transporters expression and activity. Therefore, we examined whether ceftriaxone, administered all along the hypoxic exposures, could prevent SH- and IH-induced cell death and improve tolerance to glutamate exposures.

FDA staining showed no significant difference in total number of cells in RA, IH and SH slices that were treated with ceftriaxone during their exposure (Figure 1B); However, PI-positive cells still increased after IH, compared to RA or SH albeit to a significantly lesser extent than in slices that were not treated with ceftriaxone Figure 1B. These data agreed with our LDH release results showing no significant difference in viability of slices after RA, IH or SH when slices were treated with ceftriaxone (Figure 2). Ceftriaxone prevented the increase in cell death at baseline and after each glutamate challenge in IH and SH slices, compared to their respective RA controls. Ceftriaxone treatment abrogated differences in viability between RA, IH and SH within each glutamate concentration (Figure 4). Similarly to ceftriaxone-treated slices, the first 200 μM challenge did not increase cell death in any of the conditions. However, cytotoxicity at 10 mM and after the second 200 μM challenge, while greatly attenuated by ceftriaxone, remained significantly higher compared to their respective baseline in all three experimental conditions (Fig. 4B).

These data suggest that ceftriaxone increased tolerance to glutamate and abrogated the difference in excitotoxic cell death between IH and SH-exposed slices, albeit not sufficient to completely prevent excitotoxic insult. Graphic comparison of ceftriaxone-treated and untreated slices at each glutamate concentration, revealed no significant differences in the RA or SH-exposed slices, although a clear trend of decreased excitotoxicity was apparent in ceftriaxone-treated SH slices (Fig. 5). In contrast, ceftriaxone-treated IH slices had significantly lower cell death even after 10 mM glutamate and throughout the second 200 μM glutamate challenge (Fig. 5). These data suggest that impaired glutamate transport and uptake may play an important role in IH-induced vulnerability to excitotoxic injury.

3. Effect of hypoxia on glutamate transporters expression

Our results showed that ceftriaxone reduced SH- and IH-exposed tissue liability to excitotoxicity. Therefore, to determine whether changes in EAAT1 and EAAT2 expression underlie the differences in cell viability in the various conditions, we assessed and quantified their expression in slices exposed to 7 days RA, IH or SH. Additionally, to determine whether long term SH and IH differentially affect neuronal or glial cells, MAP2 and GFAP immunoreactivity were assessed and quantified. Immunohistochemical stainings showed that EAAT1 and EAAT2 expression significantly decreased after IH and while there was a trend of decrease after SH, it did not reach statistical significance (Fig. 6). Therefore, IH appears to down-regulate glutamate transporters expression to a greater extent than SH exposure. Ceftriaxone treatment prevented EAAT1 and EAAT2 expression decrease (Fig. 7). Quantification of EAAT1 and EAAT2 immunoreactivity confirmed these results (Fig. 6). SH and IH exposure decreased GFAP and MAP2 expression compared to RA (Figures 6, 8), and ceftriaxone treatment prevented that decrease (Figures 7, 8). These results were confirmed by immunoblotting (Fig. 9). These data confirm our viability findings, showing that exposure to long term mild IH or SH significantly decreased cell viability at baseline (Figures 1, 3).

Discussion

Our results show that long-term IH, even at a moderate level of hypoxia, decreases glutamate transporters expression, resulting in cytotoxicity and reduced tolerance to glutamate exposure. In contrast, long-term moderate SH, while affecting cell viability at baseline, only minimally affected glutamate transporter expression and cell viability after glutamate challenge. These results suggest that different mechanisms may be involved in IH- and SH-induced injury that may underlie higher vulnerability to excitotoxic insult in IH-exposed slices. Indeed, we had previously reported that IH and SH induce PC12 cell apoptosis via different mechanisms [25].
The use of organotypic slices in this study, preserves the interdigitated networks of neurons and astrocytes as well as soluble factors released from neurons that are essential for the expression of GLT1 [30,31]. Brain slices are cultured at the air/media interface, directly exposed to the various gas profiles. This preparation provides adequate conditions for implementation of a physiologically relevant IH protocol allowing us to assess cellular changes that could contribute to increased vulnerability to stroke-induced excitotoxicity after exposure to various hypoxic profiles.

Preconditioning, using short bouts of ischemia or brief courses of IH, has been shown to protect against ischemic injury. IH protocols of short-term and moderate IH regimens have also been proposed in several studies as a promising therapeutic option to prevent and treat hypertension [32]. In contrast, repetitive severe and brief IH as occurs in OSA, have been shown to induce hypertension and cardiovascular disease [33]. In a recent study, Gong et al., reported IH-induced preconditioning, exposing rats to 6 h/day hypobaric hypoxia for 28 days and observed increased GLT1 expression that correlated with increased tolerance to ischemia [34]. However the IH protocol used in our study with constant repetitive IH during 7 days, rather mimics IH deleterious effects and induces a significant decrease in glutamate transporter expression. Therefore, different IH paradigms can produce remarkably divergent effects depending greatly on duration and intensity of hypoxia exposure, the number of hypoxia-reoxygenation bouts per day and the total days of the protocol.

**Figure 3. Intermittent hypoxia decreases cell viability and impairs glutamate response.** Propidium iodide staining (A) and quantification of PI positive cells presented as mean ± SD (B) of slices exposed to 7 days RA, SH or IH at baseline (BL), after 200 μM glutamate, and 10 mM glutamate, followed by a second 200 μM glutamate challenge. n = 12–18. *: At all concentrations RA < SH & IH (p<.001). #: At 10 mM: SH < IH (p<.05). 9 At 10 mM & 200 μM/#2: BL < SH & IH (p<.05 and p<.001 respectively).

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GLT1 and GLAST are sodium-dependent glutamate transporters [35]. Decreased glutamate transporter expression has been reported after acute ischemic injury, resulting in elevated extracellular glutamate [12,13]. During ischemia, the mitochondrial respiration chain is compromised, leading to energy failure that may reverse the transporter, carrying glutamate to the extracellular space and further contributing to excitotoxicity [36]. However, our hypoxic paradigm delivers a relatively mild hypoxic challenge (5% O2) that does not involve total O2 deprivation, thus moderately affects the mitochondrial respiration chain. Additionally, recurrent reoxygenation during IH preserves oxygen-dependent mitochondrial chain function preventing energy failure. Therefore, it is unlikely that reverse glutamate transport contributes to extracellular glutamate accumulation in our experimental conditions, and increased glutamate excitotoxicity in IH-exposed slices is likely due to impaired transporter expression/activity rather than to transporter reversal.

Ceftriaxone, a well-known and well tolerated β-lactam antibiotic, has been reported to induce glutamate transporter expression in rat astrocytes cultures, in organotypic spinal cord slices and in vivo in rat brain and spinal cord [21,22]. GLT1 upregulation contributes to preconditioning-induced neuroprotection against acute and delayed cell death resulting from brain ischemic injury in rats [37,38]. In agreement with these studies, ceftriaxone prevented glutamate transporters expression decrease and significantly reduced excitotoxic cell death in all three conditions also
Figure 5. Ceftriaxone effect on cell tolerance to glutamate is significantly greater in IH-exposed slices. Propidium iodide staining quantification of ceftriaxone (+) treated slices exposed to RA, SH and IH at baseline (BL), after 200 μM glutamate, and 10 mM glutamate, followed by a second 200 μM glutamate challenge. n = 12–18 for RA, SH, IH and n = 9-12 for RA+, SH+, IH+. *At BL (p = .005), †At 10 mM (p < .001) & ‡At 200 μM (p = .005).

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abrogating the increased IH susceptibility to high glutamate challenge, when compared to SH-exposed slices. While most current therapies aim at improving stroke outcome, our data suggest that ceftriaxone preserves brain glutamate response during IH exposure and could potentially serve as a prophylactic therapy to improve brain tolerance to transient ischemic events as well as minimize stroke functional consequences in a population of patients at risk.

Studies using GLT1 null mice or antisense knockdown animals have shown that GLT1 is responsible for over 90% of glutamate clearance in the CNS [19]. Our findings show that expression of both glutamate transporters decreased in IH and to a lesser extent...
in SH and that excitotoxic injury is significantly greater in IH-than in SH-exposed slices. Ceftriaxone preserved both glial and neuronal cells, prevented the decreased EAAT1 and EAAT2 expression in IH and significantly reduced excitotoxic injury in IH-exposed slices, while moderately benefiting RA- or SH-exposed slices that showed no substantial decrease in glutamate transporter expression. These data suggest that while upregulation of the transporters is beneficial in all conditions, it is critical for IH-exposed slices while additional factors may underlie excitotoxic cell death in SH-exposed slices. Our data suggest that the intermittent characteristic of IH induces distinct signaling pathways leading to a decrease in EAAT2 expression and that ceftriaxone may not only alleviate excitotoxic damage in all three conditions, but also protects from IH-induced vulnerability. The mechanism underlying IH–induced glutamate transporters down-regulation remains to be elucidated.

Several potential mechanisms could interact to decrease the expression and function of glutamate transporters in IH. At the transcriptional level, abnormal mRNA splicing results in truncated EAAT2 mRNA splice products and dysfunctional proteins [39]. Additional post-translational modifications, such as redox modulation of EAAT2 reactive amino acids may impact the transporter function [40] and may occur in IH [41,42]. Several transcription factors, such as NF-κB, upregulated during inflammation, have been shown to be regulated via redox modifications [43]. Experiments using 5'-deletion mutants of the EAAT2 promoter constructs identified one of the 4 NF-κB binding sites as a critical regulator of ceftriaxone-induced EAAT2 transcription in primary human fetal astrocytes [22,44]. Cardiovacular and cerebrovascular inflammation involving NF-κB activation during OSA has been reviewed [45,46] and has also recently been described in

Figure 8. Graphic representation of glutamate transporters, MAP2 and GFAP immunoreactivity with or without ceftriaxone. Quantification of EAAT1, EAAT2, GFAP and MAP2 immunofluorescence per unit area of slices exposed to 7 days RA, SH or IH in presence (+ve) or in absence (-ve) of 100 μM ceftriaxone. Data are presented as mean immunofluorescence ± SD. n = 4-13 for RA-, IH-, SH*; n = 5-11 for RA+, IH+, SH*. IH or SH < RA (p<0.01). #: IH* < SH* (p<0.01). doi:10.1371/journal.pone.0100230.g008

Figure 9. EAAT1 and EAAT2 immunoblotting of slices exposed to RA, SH and IH with or without ceftriaxone shows that ceftriaxone prevents IH-induced decrease in glutamate transporters. EAAT1 and EAAT2, immunoblotting of lysates from slices exposed to 7 days RA, SH or IH in absence or in presence of 100 μM ceftriaxone. Glutamate transporters expression decreased in IH-exposed slices. Ceftriaxone treatment increased EAAT1 and EAAT2 expression in all conditions and prevented EAAT1 and EAAT2 decrease in IH. doi:10.1371/journal.pone.0100230.g009
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HeLa cells exposed to IH [47] and in vivo in C57BL6 mice exposed to 14 days IH [48]. However, consequences of IH-induced NF-κB changes in the brain and their effect on glutamate homeostasis have not been investigated.

Clinical trials with glutamate receptor antagonists that would be expected to prevent excitotoxicity have been associated with untoward side effects and little clinical benefit. Thus, therapies aiming at enhancing glutamate transporter expression and activity merit exploration. Ceftriaxone, upregulating transporter expression with minimal toxicity, could be administered orally to patients at risk as a preventative therapy or during the recovery phase after a stroke. Ceftriaxone is currently in clinical trials for stroke therapy and has been shown to be neuroprotective both in vivo and in vitro in various models of acute ischemic injury [21,22], but has not been used as a prophylactic treatment in any pathological condition involving IH.

In summary, impaired glutamate transport has been implicated in multiple neurodegenerative diseases, prompting the development of novel therapeutics to increase transporters expression. However, our study presents a first report of decreased astroglial glutamate transporters as a result of a chronic, non-degenerative pathology, involving IH. Our findings suggest that preventative strategies to prevent the loss of glutamate transporters, could be implemented in such patients presenting cardiovascular alterations putting them at increased risk for stroke, and could improve their ability to withstand potential brain ischemic events.

Author Contributions
Conceived and designed the experiments: EG NMM RJ. Performed the experiments: RJ NMM EG. Analyzed the data: LRS EG. Contributed reagents/materials/analysis tools: EG NMM. Wrote the paper: RJ EG.

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