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Arctic Ground Squirrel resist peroxynitrite-mediated cell death in response to oxygen glucose deprivation

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Abstract

Cerebral ischemia-reperfusion (I/R) injury initiate a cascade of events, generating nitric oxide (NO) and superoxide ($O_2^{\bullet-}$) to form peroxynitrite ($ONOO^-$), a potent oxidant. Arctic ground squirrel (AGS; *Urocitellus parryi*) show high tolerance to I/R injury. However, the underlying mechanism remains elusive. We hypothesize that tolerance to I/R modeled in an acute hippocampal slice preparation in AGS is modulated by reduced oxidative and nitrative stress. Hippocampal slices (400 microns) from rat and AGS were subjected to oxygen glucose deprivation (OGD) using a novel microperfusion technique. Slices were exposed to NO, $O_2^{\bullet-}$ donors with and without OGD; pretreatment with inhibitors of NO, $O_2^{\bullet-}$ and $ONOO^-$ followed by OGD. Perfusates collected every 15 min were analyzed for LDH release, a marker of cell death. 3-nitrotyrosine (3NT) and 4-hydroxynonenal (4HNE) were measured to assess oxidative and nitrative stress. Results show that NO/ $O_2^{\bullet-}$ alone is not sufficient to cause ischemic-like cell death, but with OGD enhances cell death more in rat than AGS. NOS inhibitor, SOD mimetic and $ONOO^-$ inhibitor attenuates OGD injury in rat but has no effect in AGS. Rats also show a higher level of 3NT and 4HNE with OGD than AGS suggesting a greater level of injury is via formation of $ONOO^-$.

Graphical Abstract

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Conflict of interest.

The authors declare that there is no conflict of interest.

Author Contribution statement.

SB and KLD contributed to development of study design, performance of research. SB and KLD contributed to writing of manuscript. KLD contributed to financial support. All authors revised the manuscript for important intellectual content and approved the final version.

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Keywords

Hippocampal brain slices; Ischemia-reperfusion; oxygen glucose deprivation; nitric oxide; peroxynitrite; superoxide; Lactate dehydrogenase release; 3-nitrotyrosine

Introduction

Cerebral ischemia-reperfusion (I/R) injury remains an important cause of mortality and chronic morbidity in patients suffering from stroke and cardiac arrest [1]. Ischemic condition initiates a cascade of molecular events wherein ATP levels rapidly decline, arresting metabolic activity [2]. Since the maintenance of the membrane potential relies on ATP-dependent ion pumps, the cell membrane depolarizes. This causes an excess influx of calcium through voltage-gated calcium channels. As the sodium gradient dissipates, sodium-glutamate cotransporters release glutamate into the extracellular space [3], which activates postsynaptic glutamate receptors, particularly N-methyl-D-aspartate (NMDA) channels. The activated NMDA channels are responsible for a significant part of the calcium (Ca^{2+}) influx. Excessive intercellular Ca^{2+} overexcites cells and causes generation of harmful chemicals such as free radicals and reactive oxygen and nitrogen species [4].

A major event during cerebral ischemia is the generation of nitrogen and oxygen free radicals. These reactive species include superoxide ($\text{O}_2^{\bullet-}$) and nitric oxide (NO) that has the great tendency to react with each other to form a potent oxidant peroxynitrite (ONOO^-). In the brain, NO is formed by the NO synthase (NOS) isoforms, a family of enzymes [5, 6]. Under physiological conditions, NO is responsible for maintenance of basal cerebral blood flow (CBF) [7]. However, during ischemia, cerebral NO rapidly increases to 2–4 μM , producing damaging levels of NO [8, 9]. Concomitantly, a burst of free radicals such as $\text{O}_2^{\bullet-}$ [10–13] is produced after the ischemic event. This scenario is deleterious because $\text{O}_2^{\bullet-}$ anion has a high affinity for NO, higher than for the superoxide dismutase [14, 15]. NO and $\text{O}_2^{\bullet-}$ react with each other in an equimolar stoichiometric ratio forming a potent oxidant, ONOO^- further contributing to injury [16–18].

Studies have confirmed that some invertebrates and vertebrates exhibit tolerance to ischemic or hypoxic injury [19]. Ischemia causes a decrease in cerebral blood flow which in part leads

to tissue hypoxia (reduced O₂). Low O₂ causes a reduction in mitochondrial respiration and oxidative metabolism. In addition to hypoxia, decreased blood flow limits delivery of nutrients such as glucose and removal of metabolic waste such as lactate. Arctic ground squirrels (AGS), *Urocitellus parryi*, is an obligate hibernating species that tolerate cerebral I/R caused by cardiac arrest or mimicked by in vitro preparations [20–22]. Resistance to brain injury does not depend on hibernation state and persists at warm tissue temperatures [23]. Studies show that AGS hippocampus resists up to 2h of OGD [22], despite anoxic depolarization that occurs within ~ 6.6 min after onset of OGD [20]. Subsequent studies ruled out preservation of ATP levels, decreased excitatory amino acid release as mechanism of tolerance in AGS [23]. Until now, it was unclear what mechanisms, downstream of depolarization and glutamate release, contribute to AGS resistance to cerebral I/R. Here, we tested the hypothesis that tolerance to I/R injury modeled in an acute hippocampal slice preparation in AGS is modulated by reduced oxidative and nitritative stress associated with OGD.

Our results confirm that at the tissue level, AGS tolerate OGD better than ischemic susceptible Sprague-Dawley rats. The tolerance to OGD in AGS is due to less oxidative and nitritative stress marked by reduced levels of lipid peroxidation (HNE) and nitrotyrosine (3-NT) generation. The findings suggest that tolerance to OGD in AGS is modulated in part by means to mitigate oxidative and nitritative stress.

Methods

Animal Groups

Arctic ground squirrel (AGS, *Urocitellus parryi*) and Sprague-Dawley rats were used for these experiments. All procedures were approved by Institutional Animal Care and use Committee (IACUC), University of Alaska Fairbanks and performed in accordance with the guidelines of the 8th edition of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Research was conducted in accordance with ARRIVE guidelines for reporting animal research [24].

AGS of both sexes were trapped in the wild during mid-July in the northern foothills of the Brooks Range, Alaska, 40 miles south of the Toolik Field Station of the University of Alaska Fairbanks (68°38 N, 149°38 W; elevation 809 m). AGS were then transported to Fairbanks, AK under permit obtained from The Alaska Department of Fish and Game. Once transported, AGS were quarantined for two weeks and housed individually in facilities with an ambient temperature (Ta) of 16–18°C and a 12:12-h light-dark cycle. Food (rodent chow) was available *ad libitum* at all times. Experiments in AGS were conducted between July and September when AGS were in the summer euthermic state. Both males and females AGS were included in the study. Female AGS are reproductive only once a year in the spring. Neither males nor females were used during the breeding season. Male Sprague-Dawley rats (3–4 months of age at the time of the experiment) were purchased from Simonsen Laboratories (Gilroy, CA) and were transported by air to the University of Alaska Fairbanks. Rats were housed in groups of two at 20–21°C, with a 12:12-h light-dark cycle. Only male rats were used to avoid the influence of estrous on the results. Ambient temperature and light-dark cycle remained the same for all groups. AGS and Sprague-Dawley rats weighing

389 – 1036g ($701.54\text{g} \pm 37.98$) and 300 – 392g ($401.35\text{g} \pm 5.30$), respectively, were used for the study.

Acute Hippocampal slice preparation and *in vitro* modeled ischemia/reperfusion

Animals were anesthetized using 5% (v/v) isoflurane with medical grade O₂ at a constant flow rate of 1.5 L/min. Once unresponsive, the animals were euthanized via rapid decapitation and brains were removed within 2min. The whole brain was then placed in ice chilled, oxygenated HEPES buffered artificial cerebral spinal fluid (HEPES-aCSF) containing 120 mM NaCl, 20 mM NaHCO₃, 6.68 mM HEPES acid, 3.3 mM HEPES sodium salt, 5.0 mM KCl, 2.0 mM MgSO₄ (pH 7.3 – 7.4) to attenuate edema during slicing and incubation. Rapidly dissected hippocampi were embedded in 2.5 % agar and transverse hippocampal slices, 400 μm thick were cut at approximately 2°C in oxygenated HEPES-aCSF using a Vibratome® 1000plus sectioning system (The Vibratome Company, St. Louis, MO). The slices were then transferred to a brain slice keeper (Scientific Systems Design Inc., Mississauga, Ontario, CA) and allowed to recover for 1–1.5 h at room temperature (20–21°C) in HEPES-aCSF bubbled continuously with 95% O₂/5% CO₂ before transferring to microperfusion chambers.

To address the time course of injury, treatment was applied using an *in vitro* microperfusion technique described previously [25] and validated for study of modeled I/R in rats [26]. Briefly, after 1–1.5 h recovery as described above, individual slices were transferred gently to microperfusion chambers and lids sealed. The 4–8 parallel chambers (dimensions L×W×H 9×5×0.7 mm, 700 μm deep with an additional with 0.3-mm deep microchannel support each with an estimated volume of ~ 35 μL without tissue in place) were perfused with artificial cerebrospinal fluid (aCSF), pH 7.3 containing 120 mM NaCl, 45 mM NaHCO₃, 10 mM glucose, 3.3 mM KCl, 1.2 mM NaH₂PO₄, 2.4 mM MgSO₄, 1.8 mM CaCl₂ bubbled with 95% O₂/5% CO₂ and submerged in aCSF bath at 36°C ($\pm 0.2^\circ\text{C}$) at a flow rate of 7 $\mu\text{L}/\text{min}$ using Harvard Apparatus PHD 2000 syringe pump (Harvard Apparatus Holliston, MA). The osmolality of these solutions was between 290 and 300 mOsm. Sampling began 75 min after submerging the sealed chambers to allow adequate time for stabilization of neurochemical efflux.

To model *in vivo* ischemia/reperfusion (I/R)-induced alterations in the ionic microenvironment, we perfused the hippocampus slices with one of the following, (1) aCSF, pH 7.3 as a control solution, (2) OGD, pH 7.3 (glucose-oxygen free aCSF). All aCSF solutions (pH 7.3) were equilibrated with 95% O₂ and 5% CO₂ whereas the OGD solutions were equilibrated with 5% CO₂ and 95% N₂, for a minimum of 1 h until pH stabilized in the desired range. The PO₂ in OGD solution varied from 0–2.9 mmHg with an average of six determinations of 1.1 mmHg as measured using a miniature Clark-style electrode (Instech Laboratories, Plymouth Meeting, PA). The ischemia-induced alteration was made by switching the solution (from aCSF pH 7.3 to OGD pH 7.3) 8 min before the start of insult, the time it takes to completely replace the solution in the chamber with a flow rate of 7 $\mu\text{L}/\text{min}$. Perfusates were collected at 15 min intervals, and fractions were analyzed for cellular injury (LDH release) on the day of collection. Remaining volume was kept at –80°C for subsequent analysis.

Drug Treatment—To evaluate the role of NO, spermine NONOate (NO donor), or 3-bromo-7-Nitroindazole (NOS inhibitor) (Cayman Chemical) was included in the perfusion medium with and without OGD. To evaluate the role of $O_2^{\bullet-}$, hypoxanthine-xanthine oxidase (HX-XO) system was used to generate $O_2^{\bullet-}$: Bovine milk xanthine oxidase (0.4 units/mg protein) and hypoxanthine (99.0%) was obtained from Sigma. $O_2^{\bullet-}$ was decreased by the addition of the superoxide dismutase (SOD) mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL) obtained from Sigma-Aldrich Corp. FeTMPyP (Cayman Chemical), a synthetic porphyrin complexed with iron is an $ONOO^-$ decomposition catalyst and was used to decrease peroxynitrite in the microchamber.

Quantification of Cell Death

Lactate dehydrogenase (LDH) concentration in the perfusates was used as a marker of necrotic tissue damage. Fifty μ L of 105 μ L perfusates collected at 15min intervals was transferred to 384-well plates and mixed with 50 μ L reaction solution provided in the LDH assay kit (Cayman Chemical). After 60min incubation, optical density was measured at 490 nm using a microplate reader (BioTek Epoch). Background absorbance at 620nm was subtracted. The LDH release was expressed in arbitrary units per mg of protein (Biorad protein assay kit). The maximal releasable LDH was also obtained by treating the slices with 1% NP-40 lysis buffer (50 mM Tris-HCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Igepal, 150 mM NaCl, 0.05% Triton X-100) at the end of each experiment.

Determination of 4-hydroxynonenal protein adducts as a marker of lipid peroxidation

Following perfusion, each slice was homogenized in a ground glass homogenizer with 1% NP-40 lysis buffer, and the insoluble proteins were removed by centrifugation at 4°C. Protein levels in an aliquot from each supernatant were measured according to the Bradford Assay. 4-Hydroxynonenal (4-HNE) levels were then determined by competitive ELISA from aliquots of the homogenate supernatants using a 96-well OxiSelect HNE Adduct Competitive ELISA Assay Kit (Cell Biolabs®, San Diego, CA, USA) according to the manufacturer's instructions.

Determination of 3-Nitrotyrosine concentration as a marker of protein nitration

Acute hippocampal slices were homogenized on ice in 1% NP-40 lysis buffer. The slices were sonicated three times for 5 seconds each, centrifuged at 15,000g for 20 min at 4 °C. Finally, 3- nitrotyrosine (3-NT) concentrations were measured in supernatants using an OxiSelect™ Nitrotyrosine ELISA Kit (Cat. No. STA-305, Cell Biolabs, Inc., USA) according to the manufacturer's instructions. Briefly, 50 μ L of sample was added per well of ELISA plate and shaken for 10 min on an orbital shaker. Fifty μ L of anti-tyrosine antibody was then added and incubated for 1 h on an orbital shaker at room temperature. Then, each well was washed thoroughly three times with 250 μ L wash buffer. After removal of all wash buffer, 100 μ L secondary antibody-enzyme conjugate was added to all wells and incubated for 1 h at room temperature on orbital shaker. Again, each well was washed thrice with 1× wash buffer. Substrate solution (100 μ L) was added to each well and incubated for about 15 min and as color developed 100 μ L of stop solution was added per well. Immediately the plate was read at 450 nm on a spectrophotometer using a microplate reader (Epoch

Microplate Spectrophotometer, BioTek, Winooski, VT). Results were normalized per mg of protein.

Statistical Analysis

A priori power analysis (G*Power software, [27]) was performed to estimate sample size needed to yield 80% power for detecting a significant ($p < 0.05$) effect of treatment. An expected difference in means of 17.81 and standard deviations of 10.93 and 2.24, taken from rat OGD and AGS OGD indicated that a sample size of 5 slices would have 80% power for detecting a difference. No animal was excluded from the study. Slices from animals were randomly assigned to treatment and control groups without prior knowledge to the treatment conditions. Data are expressed as a fraction of baseline values. Baseline was calculated as the mean of the two samples preceding the onset of treatment. Data processing and statistical analyses were performed using Microsoft Office Excel 2010 and Prism6 (GraphPad, San Diego CA, USA). Data were analyzed with one-way or two-way ANOVA with repeated measures or t-test where indicated. Significant main effects or interactions were followed by t-tests with Bonferroni's correction. Data are expressed as mean $\pm$ SEM, and $P < 0.05$ was considered statistically significant.

Results

NO involvement in OGD injury

To illustrate that AGS tolerates OGD better than rat, hippocampal slices from a cohort of each species (rat and AGS) were exposed to 30 min OGD. Acute hippocampal slices from rat hippocampus exposed to OGD showed a significant increase in LDH release compared to control aCSF slices ($p < 0.0001$, 2-way ANOVA, treatment $\times$ time, $n = 10$ –12 slices) (Figure 1a). Slices from AGS showed less LDH release than rat in response to OGD ($p < 0.0001$, 2-way ANOVA, species $\times$ time, $n = 9$ –12 slices) (Supplementary Figure S1). Nonetheless, release of LDH in response to OGD exposure in AGS was significantly greater compared to the control aCSF slices ($p < 0.0001$, 2-way ANOVA, treatment $\times$ time, $n = 9$ –16 slices) (Figure 1d).

We next asked if NO is involved in OGD injury. The NOS inhibitor, 3-bromo-7-nitroindazole ($IC_{50} = 17 \mu M$), eliminated OGD induced cell death in rat ($p < 0.0001$, 2-way ANOVA, treatment $\times$ time, $n = 7$ –12 slices) (Figure 1a), and AGS ($p < 0.0001$, 2-way ANOVA, treatment $\times$ time, $n = 7$ –9 slices) (Figure 1d). In rat, a significant difference was also noted in total LDH release measured as the sum of LDH released from the start of insult to the end of the experiment ($p < 0.0001$, 1-way ANOVA, $n = 7$ –12 slices) (Figure 1b), however AGS showed no significant decrease in total LDH release in response to OGD ($p = 0.34$, 1-way ANOVA, $n = 7$ –9 slices) (Figure 1e).

Next, we ask if the NO donor spermine NONOate ($10 \mu M$, $T_{1/2} = 39$ min) alone caused LDH release in rat and AGS. Spermine NONOate alone did not increase total LDH release measured as the sum of LDH released from the start of insult to the end of the experiment in either rat (aCSF vs. NO donor, $p > 0.99$, 1-way ANOVA, $n = 7$ –10 slices) (Figure 1b) or AGS (aCSF vs. NO donor, $p = 0.41$, 1-way ANOVA, $n = 7$ –16 slices) (Figure 1e). A higher dose of

NO donor (40 μ M) was also tested in rat. However, even with the higher dose of NO, no statistically significant increase in injury was observed (Supplementary Figure S2). This indicates that NO donor alone is not sufficient to cause cell death. To test if exogenous NO would aggravate the OGD-induced injury, spermine NONOate was added to the OGD solution. Exogenous NO with OGD significantly increased total LDH release in rat compared to OGD alone ($p < 0.0001$, 1-way ANOVA, $n = 7-12$ slices). The response was concentration dependent with a plateau noted between 10 to 40 μ M (Figure 1c). By contrast, no significant increase in total LDH release was observed in AGS slices at concentrations up to 40 μ M ($p = 0.1$, 1-way ANOVA, $n = 7-9$ slices) (Figure 1f). These results indicate that nitric oxide contributes to OGD-induced cell death in rat and that AGS resist NO-aggravated OGD-induced death.

We further analyzed the total LDH content per slice to ask if total LDH content limits the amount of LDH release following OGD. No difference in total LDH content was observed in slices from rat or AGS that were subjected to either aCSF or OGD treatment (Supplementary Figure S3). These results suggested that the difference in LDH release between rat and AGS not be due to a difference in total LDH content but is due to a difference in the injury associated with OGD exposure. As both males and females AGS were included in the study, we also compared LDH release between males and females to see if there is a significant sex difference on LDH release when subjected to OGD. No significant differences in LDH release were observed (Supplementary Figure S4).

O₂^{•-} involvement in OGD injury

To determine the role of O₂^{•-} mediated cell death at the tissue level, acute hippocampal slices from rat and AGS were subjected to O₂^{•-} donor (HX-XO system – 0, 15, 30, 45 μ M) with and without OGD exposure for 30 min. O₂^{•-} donor exposure alone caused a slight increase in LDH release in both rat ($p < 0.0001$, 2-way ANOVA, treatment x time, $n = 7-10$ slices) (Figure 2a) and AGS ($p < 0.0001$, 2-way ANOVA, treatment x time, $n = 5-16$ slices) (Figure 2c), however, when OGD was introduced along with O₂^{•-} donor, significant increase in LDH release was observed in rat ($p < 0.0001$, 2-way ANOVA, treatment x time, $n = 7-12$ slices) (Figure 2b), but not in AGS slices ($p = 0.08$, 2-way ANOVA, treatment x time, $n = 6-9$ slices) (Figure 2d). This indicates that rat is more vulnerable to O₂^{•-} aggravated OGD-induced cell death than AGS.

Next, we ask whether O₂^{•-} causes OGD injury^{•-}. Acute hippocampal slices from both rat and AGS were pretreated with the SOD mimetic (Tempol) along with OGD. The SOD mimetic significantly attenuates OGD mediated LDH release in the rat in a concentration-dependent manner ($p < 0.0001$, 2-way ANOVA, treatment x time, $n = 7-12$ slices) (Figure 2e). In contrast, tempol pretreatment along with OGD had no significant effect on LDH release in AGS ($p = 0.46$, 2-way ANOVA, treatment x time, $n = 6-9$ slices) (Figure 2f).

We next asked if OGD injury involves ROS-mediated lipid peroxidation, a marker of oxidative stress. Results show a significant increase in total 4-HNE adduct formation in slices from a rat that was exposed to OGD as compared to aCSF treatment ($p = 0.02$, t-test, $n = 3-6$ slices). However, no significant increase in 4-HNE adduct was observed in AGS slices that were exposed to OGD ($p = 0.4$, t-test, $n = 3-7$ slices) (Figure 2g).

AGS are better protected from ONOO⁻ mediated injury than rats

Since NO is known to react with O₂^{•-} at a diffusion-limited rate to form the short-lived, potent oxidant peroxynitrite, we next examined whether ONOO⁻ was involved in OGD mediated cell death. Pretreatment of peroxynitrite decomposition catalyst 5,10,15,20-tetrakis (*N*-methyl-4'-pyridyl) porphyrinato iron III (FetMpy, 5μM) caused a significant reduction in LDH release in rat acute hippocampal slices that were exposed to OGD ($p < 0.0001$, 2-way ANOVA, treatment x time, $n = 7-12$ slices) (Figure 3a). By contrast, in AGS slices, pretreatment with the same concentration of FetMpy had no influence on LDH release caused by OGD (*refer to* Figure 1 d) ($p = 0.11$, 2-way ANOVA, treatment x time, $n = 7-12$ slices) (Figure 3b). This suggests that AGS resist peroxynitrite-mediated OGD injury.

We further examined the levels of 3-NT, a marker of peroxynitrite-mediated injury. Our results show that NO donor along with OGD increases 3-NT levels in slices from rat ($p < 0.0001$, 1-way ANOVA, $n = 6$ slices) (Figure 3c). However, AGS slices show no significant increase in 3-NT levels with increasing concentrations of NO donor along with OGD ($p = 0.07$, 1-way ANOVA, $n = 3-6$ slices) (Figure 3d). Similarly, the increases in 3-NT levels were much greater in rat slices that were subjected to increasing concentrations of the O₂^{•-} donor along with OGD ($p < 0.0004$, 1-way ANOVA, $n = 3-6$ slices) (Figure 3e). By contrast, no significant increase in 3-NT level was observed in AGS slices exposed to O₂^{•-} donor along with OGD ($p = 0.34$, 1-way ANOVA, $n = 3-5$ slices) (Figure 3f).

Discussion

Here, we tested that tolerance to I/R injury modeled in an acute hippocampal slice preparation in AGS is due to protection from oxidative and nitrative stress associated with OGD. This study shows for the first time that in isolated acute hippocampal slices, AGS resist ONOO⁻ induced lipid peroxidation and protein nitration injury during OGD. Moreover, we show that in rat both NO and O₂^{•-} are required for ischemia-modeled injury since neither alone was sufficient to produce an increase in LDH release similar in time course and magnitude to what is seen with OGD.

AGS tolerate cerebral ischemia/reperfusion caused by cardiac arrest or mimicked by in vitro preparations [21–23, 28]. AGS hippocampus resists up to 2h of OGD [22], despite anoxic depolarization [20]. Subsequent studies ruled out preservation of ATP levels or decreased excitatory amino acid release as mechanisms of tolerance [23]. Until now, it was unclear what mechanisms, downstream of anoxic depolarization and glutamate release, contribute to AGS resistance to cerebral I/R. We now know that detoxification of ONOO⁻ contributes to ischemia tolerance in AGS. We interpret the mechanism as detoxification because resistance to OGD coupled with NO was not lost at high concentrations of NO donor. We identify ONOO⁻ as the damaging species in rat because both NO and O₂^{•-} were required for injury. NO and O₂^{•-} donors paired with OGD exacerbates cell death in rat, but neither one alone is sufficient to induce cell death in rat or AGS. We interpret results to indicate that OGD generates the endogenous NO and O₂^{•-} that when combined with exogenous NO or O₂^{•-} produces ONOO⁻ and causes injury.

We know from prior studies in rat that one of the responses to cerebral ischemia is an increase in the production of NO, catalyzed by nitric oxide synthase (NOS) [29]. Recent studies demonstrated that excessive levels of NO generation due to NOS activation cause cell and tissue damage [8, 9, 29, 30]. Injury associated with NO can be related to either NO itself or be due to the interaction with $O_2^{\bullet-}$ forming $ONOO^-$ [18]. Inhibition of NOS has been reported to protect against ischemia mediated neurotoxicity [31, 32]. Our results are in accord with previous studies that inhibition of NOS reduces production of NO during OGD and significantly decreases cell death in rat [8, 33, 34]. There has been long debate as to whether NOS isoforms are activated during ischemia since the generation of NO via activation of NOS requires O_2 as a substrate [35]. However, there are substantial evidences that NO increases at 2–6 min after middle cerebral artery occlusion and in the first 10 min of OGD in the cultured hippocampus [29, 36]. NO synthesized by different NOSs has controversial effects during cerebral ischemia. Previous studies have determined that NO derived from the endothelial NOS (eNOS) is beneficial in acute ischemia. In contrast, NO produced by the neuronal NOS (nNOS) and inducible NOS (iNOS) can be neurotoxic [37]. Our approach targeted nNOS as other results showed that use of selective nNOS inhibitors, or nNOS ‘knockout’ mice, led to substantially lower infarct volumes during cerebral ischemia [8]. By contrast, iNOS becomes upregulated from 12 hours after middle cerebral artery occlusion (MCAO) for up to 7 days [38]. For this reason, we used an inhibitor of nNOS 3-bromo-7-nitroindazol (0.17 μ M) (IC_{50} = 0.17 μ M). However, because 3-bromo-7-nitroindazol inhibits iNOS (IC_{50} = 0.29 μ M) and eNOS (IC_{50} = 0.86 μ M) [39] at slightly higher concentrations, we cannot rule out involvement of eNOS and iNOS isoforms. Resistance to OGD in AGS did not depend on lack of NOS activation since NOS inhibition eliminated the small amount of OGD-induced injury in AGS. Although this injury was much less in AGS than in rat, both were eliminated by NOS inhibition, so both involved NOS activation.

Increase in LDH release when exogenous NO is paired with OGD but not alone also reflects that NO combines with a reactive species generated during OGD that together contributes to cell death. In our study, a nitric oxide donor (spermine NONOate) was chosen as an exogenous source of NO due to its simplicity in handling, storage stability and its convenient half-life in solution (about 39 min at 37°C). Spermine NONOate decomposes in solution producing 2 moles of NO per mole of the parent compound. In our microperfusion approach, the time taken for the perfusion solution containing spermine NONOate to reach the slice is 8 min. We therefore, do not know the actual concentration of NO in the slice, but are confident based on observations that spermine NONOate exacerbated OGD-induced injury in rats that the concentration and time of NONOate to reach the slice was sufficient to increase NO levels in the slice.

Generation of $O_2^{\bullet-}$ could be limited during OGD due to decreased availability of O_2 . We do not think this is the case because other results support that $O_2^{\bullet-}$ generated and monitored during OGD in rat is cytotoxic [40] and cell death during OGD is attenuated by a SOD mimetic (Tempol) [41]. Tempol is a membrane-permeable SOD mimetic that catalyzes the reduction of $O_2^{\bullet-}$ to H_2O_2 [42] and can react with both extra- and intra-cellular O_2 free radicals showing neuroprotective effects [43]. As an SOD mimetic, Tempol metabolizes $O_2^{\bullet-}$ generated in solutions of xanthine plus xanthine oxidase [44] and is neuroprotective

against $O_2^{\bullet-}$ and $ONOO^-$ -induced inflammation [45]. Recently the specificity of Tempol for $O_2^{\bullet-}$ has been questioned since it also reduces the formation of hydroxyl radicals [46, 47] and attenuates the cytotoxic effects of H_2O_2 [48]. Tempol also inhibits $ONOO^-$ -mediated nitration of tyrosine [49]. The lack of specificity of tempol to reduce $O_2^{\bullet-}$ does not change our interpretation that $O_2^{\bullet-}$ alone is not the damaging species during OGD. We base this conclusion on the observation that the generation of $O_2^{\bullet-}$ by hypoxanthine plus xanthine oxidase produced a negligible increase in cell death. Only when combined with OGD did $O_2^{\bullet-}$ produce pronounced ischemic-like cell death and OGD-induced cell death was blocked by Tempol.

During I/R injury, three distinct mechanisms generate ROS contributing to cell death: mitochondrial ROS generation, xanthine oxidase (XO) system and the NADPH oxidase complex. The paradox of increased $O_2^{\bullet-}$ production during ischemia is counterintuitive since O_2 levels are decreased during ischemia, and ROS generation increases during the reperfusion phase when there is a high influx of O_2 . However, the rate of $O_2^{\bullet-}$ generation is affected both by O_2 level and the availability of reduced flavins and quinones as a source for univalent electron transfer. The tendency of reduced flavins to increase $O_2^{\bullet-}$ production at lower O_2 concentrations was previously described [50]. During ischemia, mitochondrial electron transport slows, increasing the reduction state of electron carriers. This favors $O_2^{\bullet-}$ generation if some O_2 is still available. Our approach shows that an SOD mimetic (Tempol) attenuates OGD induced cell death suggesting that during OGD there is generation of $O_2^{\bullet-}$ which is in accord with previous work showing that increased mitochondrial ROS generation occurs during the first few minutes of hypoxia [51]. Increase in LDH release in rat when $O_2^{\bullet-}$ donor was paired with OGD also support that $O_2^{\bullet-}$ contributes to $ONOO^-$ mediated cell death whereas AGS resist this scenario. In our study, HX-XO system was used to generate $O_2^{\bullet-}$ although under most conditions xanthine oxidase generates more H_2O_2 than $O_2^{\bullet-}$ [52]. For this reason, we cannot rule out that H_2O_2 may react with a species generated during OGD. This is less likely to be the fate of H_2O_2 , however, because H_2O_2 is less reactive than $O_2^{\bullet-}$ [53]. $O_2^{\bullet-}$ generated during ischemia undergoes rapid dismutation to H_2O_2 by SOD. However, since NO is also generated during OGD, the presence of NO in close proximity to $O_2^{\bullet-}$ favors the formation of $ONOO^-$ over H_2O_2 since the rate constant of NO with $O_2^{\bullet-}$ is larger than with SOD and therefore NO outcompetes SOD for $O_2^{\bullet-}$ [54, 55]. This mechanism explains why $O_2^{\bullet-}$ formation by xanthine oxidase is sufficient to mediate alterations in vascular function by reducing NO bioavailability via direct reaction ($NO + O_2^{\bullet-} \rightarrow ONOO^-$) [56].

Our results support a dominant role for $ONOO^-$ in OGD-induced injury because when FeTMPyP, a peroxynitrite decomposition catalyst (PDCs) was used, LDH release was significantly reduced in rat. FeTMPyP exhibits good specificity as a peroxynitrite decomposition catalyst with an EC50 value of 3.5 μM [57], and does not influence NO or $O_2^{\bullet-}$ action [57–60]. We used FeTMPyP at a concentration of 5 μM as this concentration is near the EC50 value and was shown previously to be efficacious in $ONOO^-$ decomposition [61, 62]. Furthermore, rat slices subjected to OGD in presence or absence of NO/ $O_2^{\bullet-}$ increases 4-HNE adduct and 3-nitrotyrosine level. Both 4-HNE adduct and 3-nitrotyrosine are generated in the presence of $ONOO^-$ [63, 64]. Although the biomolecular modifications are not specific markers of $ONOO^-$ and the Elisa kits may not be specific for 3NT or HNE,

the results are consistent with the presence of ONOO^- and in light of other results discussed above support the interpretation that injury is due to ONOO^- .

Our results support that the increase in cell death is due to a direct bimolecular reaction of NO with $\text{O}_2^{\bullet-}$ generated during OGD which others have shown to yield ONOO^- at almost diffusion-limited rates (rate constant (k) = $6.7 - 19 \times 10^9/\text{M/s}$) [17, 65]. By rapidly consuming $\text{O}_2^{\bullet-}$, NO produces $\text{ONOO}^-/\text{ONOOH}$. ONOO^- is a potent oxidant that plays a critical role in neurotoxicity of NO and $\text{O}_2^{\bullet-}$ [66]. ONOO^- can easily cross the plasma membrane and oxidize many intracellular molecules including lipids, DNA and proteins [67], subsequently inducing protein dysfunction and DNA damage, and cell death [68]. Previous studies also indicate that ischemia causes an increase in levels of NO, $\text{O}_2^{\bullet-}$ and ONOO^- concentrations in the brain from basal levels of 1–10 nM [69] to the low μM range [70] with subsequent protein nitration and cellular injury [71]. Our results are consistent with other studies suggesting that excess NO radical in the presence of $\text{O}_2^{\bullet-}$ is neurodestructive [72]. Other studies in human neuroblastoma cells also reported that when NO donor was applied along with nontoxic concentrations of H_2O_2 (an oxidative stress), neurotoxicity due to the formation of ONOO^- was observed [73]. Resistance to cell death in AGS associated with NO or $\text{O}_2^{\bullet-}$ donor with OGD suggests that AGS avoids this mechanism of injury that is deleterious in rat. Pretreatment with FeTMPyP during OGD further confirms that ONOO^- is generated during OGD [74] causing lipid peroxidation and protein nitration, markers of ONOO^- mediated cell death [63]. Lower levels of lipid peroxidation and protein nitration in AGS suggest that AGS resist ONOO^- induced cell death. Any mechanism involving NO/ ONOO^- , must however, remain speculative since we have not measured actual NO, $\text{O}_2^{\bullet-}$ and/or ONOO^- levels.

The microperfusion technique [25] used in the present study was developed specifically for the study of OGD in adult brain slices. Constant perfusion of slices with aCSF maintains slice integrity for ~8 hours. This approach was used previously to monitor glutamate and adenosine release in rat slices during OGD [26]. We acknowledge that the in vitro approach used for our study might not apply to the in vivo scenario wherein whole animal mechanisms such as pH, buffering capacity and limited inflammatory response contribute to tolerance to ischemia reperfusion injury [75]. However, this approach isolates mechanisms intrinsic to hippocampus and allows for each component to be manipulated in the ischemic cascade to study mechanisms at the tissue level. The approach allows for the study of cell death in adult AGS tissue. Although the time constraint in tissue viability in our approach limits the study of apoptotic cell death that is possible with organotypic brain slice culture, capacity to study adult tissue is an important advantage since AGS are seasonal breeders which limits availability of neonatal tissue for organotypic culture [76]. Another advantage with this approach is that cell death can be monitored by capturing LDH release [77] from the entire slice and thus overcomes the limitations of methods used in prior studies where high baseline cell death could have compromised interpretation [21, 22]. Finally, the method provides some temporal resolution to study the time course of cell death.

Our experimental approach reduces animal numbers and refines techniques as per the arrive guidelines. We have chosen the slice model for our study because brain tissue is a complex collection of neurons and glia that cannot be modeled entirely using cell culture. Cell and

tissue culture differ from adult brain slices in several ways such as the influence of age (adult vs. neonatal) on the mechanisms of resistance, the proportion of neurons to glia, synaptic connections between neurons, neural circuitry that affects metabolic demand of the tissue and maturity of the neurons studied. However, the slice model reduces the number of animals used and refines the experimental approach to minimize animal pain and suffering associated with whole animal studies. The microperfusion technique also refines the approach to the study of brain injury by providing a time course of acute injury that has not been possible to date with cell and tissue culture.

Conclusions

In summary, our data supports that NO and $O_2^{\bullet-}$ alone are not causing ischemic-like injury in rat or AGS because neither produced a large amount of injury except when paired with OGD. OGD injury is caused by the release of both NO and $O_2^{\bullet-}$ and one without the other is not sufficient to induce ischemic-like cell death. Our data suggests that the ischemic-like injury is due to the combination of NO and $O_2^{\bullet-}$ together forming ONOO⁻ leading to cell death. We interpret these findings to mean that resistance to injury in AGS is associated with reduced susceptibility to highly reactive ONOO⁻ generated during I/R injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- AGS hippocampal slices tolerate oxygen glucose deprivation (OGD) better than rat.
- OGD injury is attenuated by both nNOS inhibitor and SOD mimetic indicating both NO and $O_2^{\bullet-}$ plays a role in injury.
- NO and $O_2^{\bullet-}$ alone is not sufficient to induce ischemic-like cell death.
- Both NO and $O_2^{\bullet-}$ donor exacerbates OGD induced injury in rat but no effect in AGS.
- AGS tolerate $ONOO^-$ mediated injury better than rat.

Figure 1. AGS resist nitric oxide mediated LDH release

(a, d) Slices from rats and AGS were subjected to 30 min of aCSF, OGD, and OGD + NOSI. The time course of LDH release is shown. * $p < 0.05$ (aCSF vs. OGD), # $p < 0.05$ (OGD vs. OGD + NOSI). (b, e) Total LDH (the sum of LDH released from the start of insult to the end of the experiment) is shown in a and b. * $p < 0.05$ vs. aCSF, + $p < 0.05$ vs. NO (10 μ M), # $p < 0.05$ vs. OGD + NOSI. (c, f) Total LDH release from additional experiments in which NO donor was included with OGD. * $p < 0.05$ vs. OGD. Grey bar indicates insult period.

Figure 2. AGS show better tolerance to $O_2^{\bullet-}$ mediated cell death

(a, c) Acute hippocampal slices from both rat and AGS were subjected to different concentrations of $O_2^{\bullet-}$ donor (HX-XO: 0, 15, 30, 45 mU/ml) and LDH release over a course of time was monitored. * $p < 0.05$ (0mU/ml vs. 45mU/ml), + $p < 0.05$ (0mU/ml vs. 30mU/ml). (b, d) Time course of LDH release was monitored in rat and AGS slices exposed to different concentrations of $O_2^{\bullet-}$ donor (HX-XO: 0, 15, 30, 45 mU/ml) along with OGD. * $p < 0.05$ (OGD+0mU/ml vs. OGD+45mU/ml), + $p < 0.05$ (OGD+0mU/ml vs. OGD+30mU/ml). (e, f) Acute hippocampal slices from rat and AGS pretreated with SOD mimetic (Tempol) followed by OGD. * $p < 0.05$ (OGD vs OGD+Tempol (3mM)), + $p < 0.05$ (OGD vs OGD +Tempol (0.03mM)) (g) shows total 4-HNE-Protein adduct formation in rat and AGS slices exposed to OGD * $p < 0.05$ vs. aCSF.

Figure 3.

AGS tolerate peroxynitrite mediated OGD injury. (a, b) rat and AGS slices were preincubated with FetMpyr (peroxynitrite decomposition catalyst) and LDH release was monitored. * $p < 0.05$ vs. OGD. (c, d) 3-NT was measured in rat and AGS slices subjected to NO donor along with OGD. * $p < 0.05$ vs. OGD, + $p < 0.05$ vs. aCSF. (e, f) 3-NT was measured in rat and AGS slices when subjected to $O_2^{\bullet-}$ donor along with OGD. * $p < 0.05$ vs. OGD.