

Cellular mechanisms by which lipoic acid confers protection during the early stages of cerebral ischemia: A possible role for calcium

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ARTICLE INFO

Article history:

Received 19 October 2010

Received in revised form

29 November 2010

Accepted 17 December 2010

Available online 24 December 2010

Keywords:

Lipoic

Neuroprotection

Stroke

Electrophysiology

OGD

Calcium

ABSTRACT

Lipoic acid (LA) is a naturally occurring compound and dietary supplement with powerful antioxidant properties. Although LA is neuroprotective in models of stroke, little is known about the cellular mechanisms by which it confers protection during the early stages of ischemia. Here, using a rat model of permanent middle cerebral artery occlusion (MCAO), we demonstrated that administration of LA 30 min prior to stroke, reduces infarct volume in a dose dependent manner. Whole-cell patch clamp techniques in rat brain slices were used to determine if LA causes any electrophysiological alterations in either healthy neurons or neurons exposed to oxygen and glucose deprivation (OGD). In healthy neurons, LA (0.005 mg/ml and 0.05 mg/ml) did not significantly change resting membrane potential, threshold or frequency of action potentials or synaptic transmission, as determined by amplitude of excitatory post synaptic currents (EPSCs). Similarly, in neurons exposed to OGD, LA did not alter the time course to loss of EPSCs. However, there was a significant delay the onset of anoxic depolarization as well as in the time course of the depolarization. Next, intracellular calcium (Ca^{2+}) levels were monitored in isolated neurons using fura-2. Pretreatment with 0.005 mg/ml and 0.05 mg/ml LA for 30 min and 6 h did not significantly alter resting Ca^{2+} levels or Ca^{2+} response to glutamate (250 μM). However, pretreatment with 0.5 mg/ml LA for 6 h significantly increased resting Ca^{2+} levels and significantly decreased the Ca^{2+} response to glutamate. In summary, these findings suggest that LA does not affect neuronal physiology under normal conditions, but can protect cells from an ischemic event.

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1. Introduction

Lipoic acid (LA) is a naturally occurring compound commonly found in diet but which is also endogenously synthesized in small amounts by plants and animals, including humans (Reed, 2001; Choi et al., 2009). An essential co-factor for aerobic metabolism (Bilska and Wlodek, 2005), LA is also a potent free radical scavenger, antioxidant (Packer et al., 1997; Somani et al., 2000; Wang and Chen, 2007) and a powerful anti-inflammatory agent (Sola et al., 2005). Because of these physiological functions, synthetically produced LA is widely given as a dietary supplement and has been extensively investigated for its therapeutic potential. Lipoic acid has been demonstrated to have many diverse therapeutic

uses including, but not limited to, the treatment of diabetes and diabetic neuropathy (Singh and Jialal, 2008; Poh and Goh, 2009), treatment of liver (Bustamante et al., 1998) and cardiovascular (Ghibu et al., 2009) diseases and treatment of Alzheimer's disease (Maczurek et al., 2008). As well, LA has been demonstrated to reduce the risk factors associated with stroke. For example, it decreases LDL (Zulkhairi et al., 2008), increases weight loss (Ying et al., 2010), and reduces blood pressure (Vasdev et al., 2003). Furthermore, it has been demonstrated that LA can directly reduce brain damage caused by stroke (Panigrahi et al., 1996; Prehn et al., 1992; Wolz and Kriegelstein, 1996). Much of the research in this area has focused on the neuroprotective ability of LA during reperfusion (restoration of blood flow to an ischemic area following a stroke; Panigrahi et al., 1996; Clark et al., 2001). Events seem to suggest that a potential mechanism of this neuroprotection involves the scavenging of free radicals, which largely account for the cell death following reperfusion (Panigrahi et al., 1996; Packer et al., 1997). Interestingly, despite the attention given to the neuroprotective ability of LA during reperfusion, little is known about the beneficial effects of LA on the early cellular events that take place following the onset of ischemia. During this phase of a stroke, free radical damage is less important than during reper-

Abbreviations: aCSF, artificial cerebrospinal fluid; AD, anoxic depolarization; APs, action potentials; EPSC, excitatory postsynaptic currents; GABA, gamma aminobutyric acid; LA, lipoic acid; MCAO, middle cerebral artery occlusion; NMDA, N-methyl D-aspartate; OGD, oxygen-glucose deprivation; RMP, resting membrane potential; TTC, tetrazolium chloride; V_h , holding potential.

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fusion and thus any neuroprotective effects of LA, if any, are not likely due to this compound's antioxidant abilities. Thus, the purpose of this study was to investigate the cellular mechanisms by which LA may confer protection during the early stages of cerebral ischemia, during development of the ischemic core. Specifically, there were three main aims: (1) to determine if LA could be neuroprotective in an *in vivo* model of permanent occlusion, (2) to determine if LA pretreatment results in changes in neuronal excitability under normal or ischemic conditions *in vitro*, and (3) to determine if LA pretreatment causes changes in basal intracellular Ca^{2+} levels or changes in glutamate evoked Ca^{2+} release, either of which may suggest a potential mechanism by which LA confers neuroprotection.

2. Materials and methods

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care and under an approved protocol by the University of Prince Edward Island Animal Care Committee.

2.1. Permanent middle cerebral artery occlusion (MCAO) *in vivo*

All experiments were conducted on male Sprague-Dawley rats (24 rats; 200–350 g; Charles Rivers; Montreal, PQ, CAN). For all animals, food and tap water were available *ad libitum*. Rats were anaesthetized with sodium thiobarbital (Inactin; RBI, Natick, MA, USA; 100 mg/kg; ip) which provided a stable plane of anesthesia for the full duration of the experiment (no animals required anesthetic supplementation). A polyethylene catheter (PE-50; Clay Adams, Parsippany, NJ, USA) was inserted into the right femoral artery to monitor blood pressure and heart rate, and a second polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was inserted into the right femoral vein to permit intravenous administration of drugs. Arterial blood pressure was measured with a pressure transducer (Gould P23 ID, Cleveland, OH) connected to a Gould model 2200S polygraph (Gould, Cleveland, OH). Heart rate was determined from the pulse pressure using a Gould tachograph (Biotach; Gould, Cleveland, OH). These parameters were displayed and analyzed offline using PolyviewPro/32 data-acquisition and analysis software (Grass; Warwick, RI). An endotracheal tube was inserted to facilitate breathing. Body temperature was monitored and maintained at $37 \pm 1^\circ\text{C}$ using a Physiotemp feedback temperature control system (Physiotemp Instruments; Clifton, NJ).

For the permanent occlusion of the middle cerebral artery (MCA), techniques were identical to those previously published from our laboratory (Connell and Saleh, 2010). Briefly, animals were placed in a David Kopf stereotaxic frame (Tujunga, CA, USA) and the right MCA approached through a rostra-caudal incision of the skin and frontalis muscle at the approximate level of bregma. Blood flow through the MCA was impeded by the placement of surgical suture behind the MCA at 3 designated positions along the exposed vessel. The ends of these sutures were positioned so that the middle of the sutures applied pressure to the MCA from behind and impeded blood flow through the MCA. This 3-point placement of surgical sutures produced a highly reproducible and consistent focal ischemic lesion volumes restricted to the ipsilateral cerebral cortex (Connell and Saleh, 2010).

Following 6 h of permanent MCA occlusion (MCAO), all animals were perfused transcardially with phosphate buffered saline (PBS; 0.1 M; 200 ml), the brains removed and sliced into 1 mm coronal sections using a rat brain matrix (Harvard Apparatus; Holliston, MA, USA). Sections were incubated in a 2% solution of 2,3,5-triphenol tetrazolium chloride (TTC; Sigma-Aldrich; St. Louis; MO, USA) for 5 min. Infarct volumes were calculated with the use of

scanned digital images of each brain section. The infarct area for both sides of each brain section was calculated using a computer-assisted imaging system (Scion Corporation; Frederick, MD, USA). The infarct areas for each side for each individual section were averaged and multiplied by the width of each section (1 mm) to give the infarct volume for each section. The sum total of all the individual infarct volumes provided the infarct volume for each rat.

2.2. *In vitro* brain slice preparation

In vitro focal ischemia studies were performed on freshly prepared rat brain slices. Methods for preparing the brain slices were similar to those previously published (Saleh et al., 1997). Briefly, male Sprague-Dawley rats (124–150 g; Charles River, Montreal, PQ, Canada) were anaesthetized with isoflurane vapor (IsofloTM; Abbott Laboratories, Saint-Laurent, PQ) and then decapitated. Brains were rapidly removed and immersed in ice-cold ($2\text{--}3^\circ\text{C}$) artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 145 NaCl, 2.5 KCl, 10 D-glucose, 26 NaHCO_3 , 1.2 NaH_2PO_4 , 1.3 MgCl_2 , 2.5 CaCl_2 (pH 7.4, osmolality of 295–305 mOsm/L, continuously bubbled with 95% O_2 and 5% CO_2). The brain was then mounted in a vibratome (VT 1000S, Leica) and coronal sections (400 μm thick) were sliced while submerged in ice-cold aCSF. Prior to the initiation of experiments, slices were incubated for at least 1 h in aCSF at room temperature.

For experimentation, an individual slice was transferred to an experimental chamber and viewed under low magnification using an upright microscope (BX51WI, Olympus Canada Inc., Markham, ON, Canada). aCSF was superfused at 3 ml/min and the bath temperature was continuously monitored and maintained at $35 \pm 1^\circ\text{C}$ using a temperature control unit (TC-344B, Warner Instruments, Hamden, CT, USA). Bath level was controlled and perfusate was removed via a suction tube located at the opposite end to the inflow of the chamber. The slice was suspended on a mesh insert placed within the bath and was secured in place by a slice anchor hold-down apparatus (thin wire loop with fine thread spaced approximately 1 mm apart). Ischemia was induced by bath perfusion of oxygen–glucose deprived (OGD) medium. The composition of this OGD medium was the same as that of the aCSF solution except that glucose was excluded and replaced with 10 mM D-mannitol and that oxygen was displaced (verified using an O_2 sensing electrode) by bubbling continuously with 95% N_2 and 5% CO_2 .

2.3. Primary cortical cultures

Primary neuronal cultures were isolated using methods described previously (Chan et al., 2000). Briefly, pregnant females were sacrificed at embryonic day 16 and the embryos were removed and placed on ice. Cerebral cortices were dissected from fetuses and placed in Hank's balanced salt solution (HBSS, Gibco). HBSS was removed and tissue was resuspended in warm trypsin solution (0.25% in HBSS, Invitrogen), and incubated for 15 min at 37°C . Cells were dissociated using a large bore 1000 μl pipette tip then were resuspended in neurobasal medium (Gibco) supplemented with 2% B27 (Gibco), 5% fetal bovine serum (Gibco) and 1% Gentamycin (Gibco). Cells were seeded onto poly-D-lysine-coated glass coverslips at a density of 0.8×10^6 cells per well in 24-well plates and cultured at 37°C with 5% CO_2 . After 24 h media was replaced with serum-free neurobasal medium supplemented with B27 and gentamycin, and half the media was replaced with fresh media every 3 days. Experiments were performed following 5–9 days of culture.

2.4. Electrophysiological experiments

“Blind” whole-cell patch-clamp recordings (Blanton et al., 1989) were performed on cortical neurons using a MultiClamp 700B amplifier with CV-7B head stage (Axon Instruments, Union City, CA, USA). Patch pipettes (5–8 M Ω ; KG-33; Garner Glass Co., Claremont, CA, USA) were pulled with a Flaming-Brown micropipette puller (Model P-87; Sutter Instruments Co., Novato, CA, USA). Patch pipette filling (internal) solution consisted of the following (in mM): 130 K-gluconate, 6 NaCl, 10 HEPES, 2.5 Na-ATP, 0.1 Na-GTP; pH 7.3 with KOH, osmolarity of 285–295 mOsm/L. Before breaking into the cell with suction, fast electrode capacitance was compensated. Electrophysiological criteria for a neuron to be used in an experiment were based on their excitability, having a starting resting membrane potential (RMP) more negative than –60 mV, and an access resistance ranging between 15 and 35 M Ω . Data acquisition and analysis were performed using Clampex and Clampfit 9.2 software, respectively (Axon Instruments, Union City, CA, USA). All data were acquired at a sampling rate of 10 kHz.

In experiments where changes to RMP were measured, neurons were current clamped ($I = 0$) and continuous recordings were made using Pclamp's gap-free mode. Recordings of action potentials were generated in current clamped neurons by continuous current injection for 200 ms (50 pA steps from 0 pA). Threshold current was defined as the minimal amount of current required to elicit an action potential. Excitatory post synaptic currents (EPSCs) were isolated using bicuculline (25 μ M) and were recorded as inward currents from voltage clamped neurons ($V_h = -65$ mV). EPSCs were evoked using an ISO-Flex stimulus isolator (A.M.P.I., Jerusalem, Israel) connected to a matrix tungsten electrode (FHC Inc., Bowdoin, ME, USA) placed in layer 2 of the cortex. Recordings were made from neurons in layer 5 of the cortex adjacent to the stimulating electrode. All cells had a graded evoked response to increased stimulus intensity, and an intensity giving a 70–80% of the maximum evoked synaptic response was used to evoke test responses. Synaptic responses were elicited at 30 s intervals.

2.5. Single cell Ca^{2+} imaging

Cortical neuronal cells were loaded for 30 min at 37 °C in HEPES-buffered saline solution (HBS; 119 mM NaCl, 25 mM HEPES, 2.5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, and 30 mM glucose, pH 7.4) containing fura-2 AM (4 μ M, Invitrogen) and pluronic F-127 (0.1%). Coverslips of cells were washed with HBS then incubated for a further 15 min. Individual coverslips were then placed in a laminar flow chamber (FCS2; Biopetech, Butler, PA, USA) mounted on an inverted epifluorescence microscope (Axiovert 200; Zeiss) equipped with a CCD digital camera (Axiocam MRm; Zeiss). Perfusion solutions were gravity fed at 2 ml/min and were interchanged using solenoid valves (Valvelink 8 controller, Automate Scientific, Berkeley, CA, USA). Slidebook software (Intelligent Imaging Systems, Denver, CO, USA) controlled excitation light from a Lambda DG-4 unit (Sutter Instrument, Novato, CA, USA) equipped to provide alternating excitation at 340 and 380 nm. Emitted light was collected through a custom dichroic block for fura-2 (Chroma, Rockingham, VT, USA). The rate of image acquisition was varied for different experiments and was controlled by the Slidebook software. Ca^{2+} responses were quantified by placing regions of interest on 15–20 neurons within the field of view and calculating the ratio between measured excitation at 340 nm vs. 380 nm at each time point.

2.6. Data analysis and statistics

Measurements of RMP were taken as an average over 10 s. EPSC amplitudes (at V_h of –65 mV) were measured from baseline to

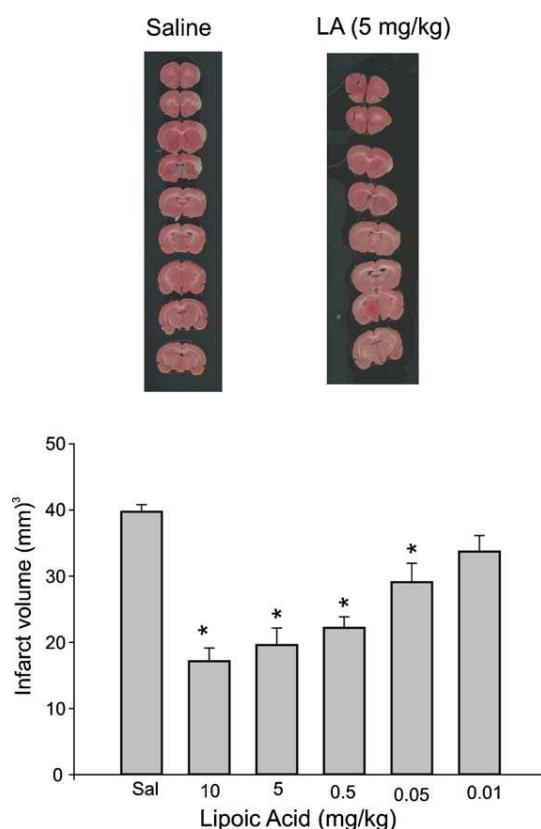


Fig. 1. LA reduces infarct volume in an *in vivo* rat model of permanent middle cerebral artery occlusion (MCAO). (A) Representative TTC stained images taken from rats treated with either saline or lipoic acid (LA; 5 mg/kg) on infarct volume in 1 mm coronal slices throughout the brain following the 6 h of permanent MCAO. (B) Bar graph of the average infarct volume in rats treated with either saline as vehicle (sal), or LA at concentrations from 10–0.01 mg/kg (each bar represents the average from 4 rats). Each data point represents mean $\pm$ SEM ($n = 4$) and (*) indicates significant difference ($p < 0.05$) compared to the saline group (ANOVA).

peak. Percentage changes in responses were calculated by dividing the amplitude of each response by the averaged baseline response multiplied by 100 then subtracting 100.

All data are presented as mean $\pm$ standard error of the mean (SEM). Unless stated otherwise, differences between treatment groups at identical time points were determined using two-way ANOVA followed by a Student–Newman–Keul's post hoc test. Differences in latency of anoxic depolarization between treatment groups were determined using an unpaired Student's *t*-test. Statistics were performed using SigmaStat software (Systat Software Inc., San Jose, CA, USA). In all cases, differences were considered significant if $p \leq 0.05$ and (n) represents the number of recorded cells that were included in the study. Only one cell was recorded from within each slice.

2.7. Drugs and solutions

All laboratory salts were purchased from Fisher Scientific Canada (Ottawa, ON, Canada). α -Lipoic acid and glutamate was purchased from Sigma–Aldrich Canada Ltd. (Oakville, Ontario, Canada). Stock solution of lipoic acid and glutamate were made up in physiological saline solution (to a pH of 7.4). Aliquots of these stocks were added to the perfusion media to the final concentrations indicated. Solvent controls were performed when necessary.

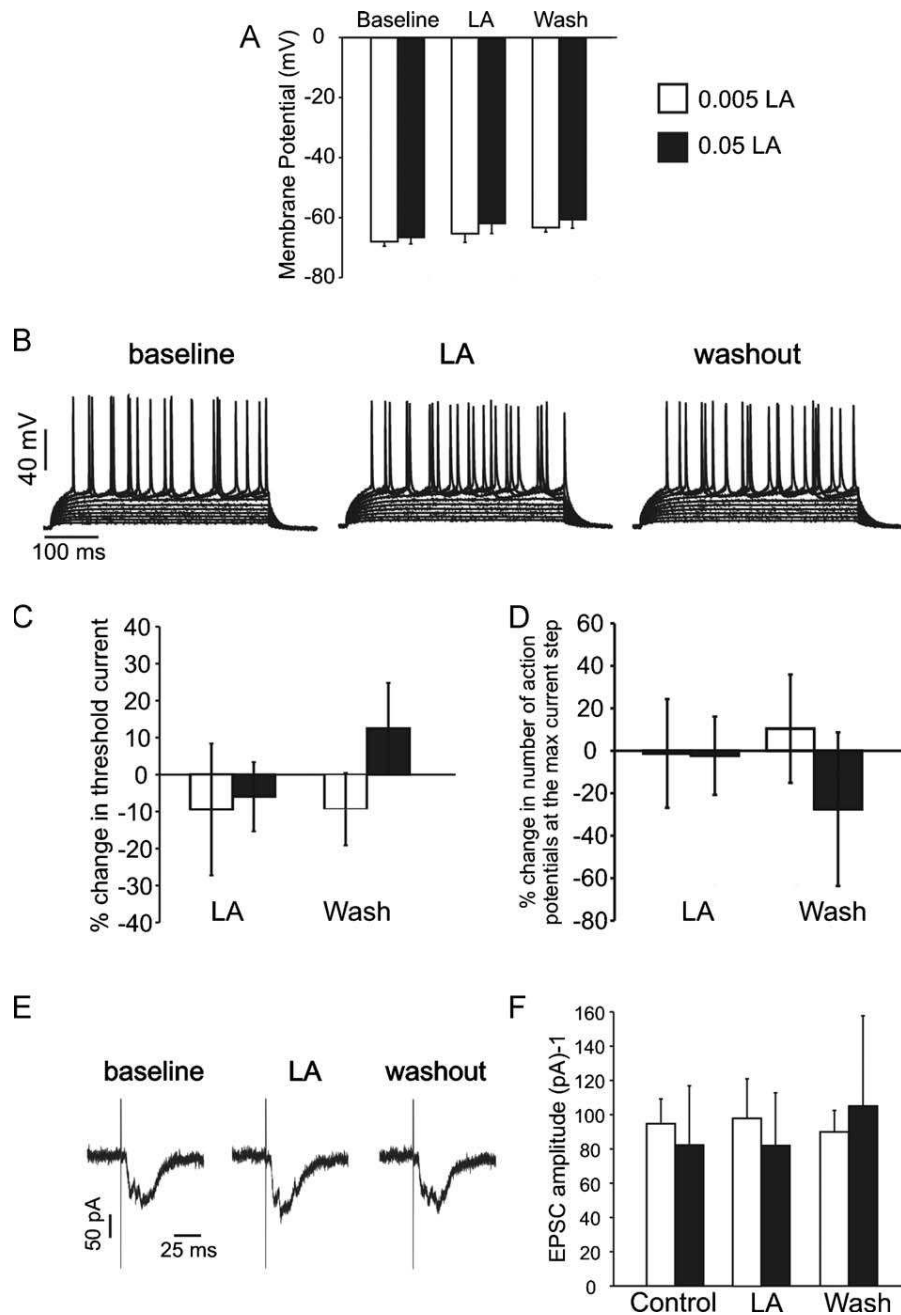


Fig. 2. Effect of LA on electrophysiological parameters of healthy neurons. Recordings were made using whole cell patch clamp techniques. Following a 5 min baseline recording in aCSF, the media was switched to aCSF + LA (0.005, 0.05 mg/ml) for 30 min then switched back to aCSF for a 10 min washout period. (A) Graphical representation of neuronal resting membrane potential during baseline, at the end of the LA treatment and at the end of a washout period. (B–D) Effect of LA on the generation of action potentials. (B) Representative intracellular recording of action potentials during baseline, during LA treatment, and during washout. The protocol used to generate the AP (top) consisted of 200 ms continuous current injections (50 pA steps from 0 pA). (C) Graphical representation of the effect of LA application on the minimal amount of current required to elicit an action potential (threshold). Data are expressed as percentage change from baseline. (D) Graphical representation of the effect of LA application on the number of action potentials generated by the maximal current step. The maximal current step was kept constant within any given experiment, but may have varied between different experiments. Data are expressed as percentage change from baseline. (E and F) Effect of LA application on excitatory post synaptic potential (EPSC) amplitude. (E) Representative recordings of EPSCs during baseline, LA treatment, and during washout. (F) Graphical representation of the effects of LA treatment on EPSC amplitude. Each data point represents mean $\pm$ SEM ($n=3$).

3. Results

3.1. Effect of LA on permanent occlusion model

Pretreatment of animals with LA, 30 min prior to MCAO resulted in a dose-dependent decrease in infarct size (Fig. 1A and B). Vehicle treated animals ($n=4$) had an average infarct volume of $39.5 \pm 2 \text{ mm}^3$. Animals pretreated with LA at 10, 5, 0.5 and 0.05 mg/ml significantly reduced infarct volume to $18 \pm 3 \text{ mm}^3$,

$20 \pm 4 \text{ mm}^3$, 23 ± 3 and $29 \pm 4 \text{ mm}^3$, respectively ($n=4/\text{group}$; $p < 0.05$; ANOVA).

3.2. Effect of LA on electrophysiological parameters of healthy neurons

The following set of experiments were conducted to determine whether changes in membrane excitability contributed to

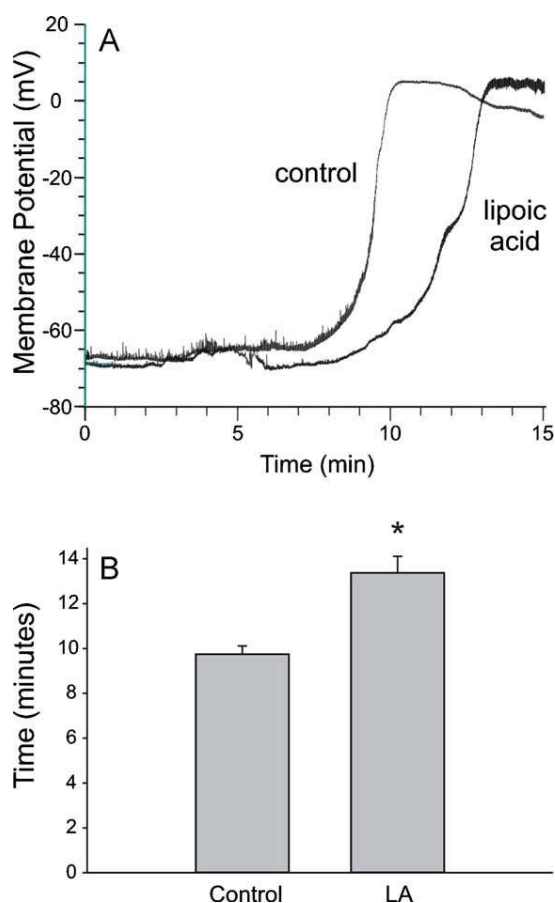


Fig. 3. Effect of LA on the latency to anoxic depolarization under ischemic conditions. Intracellular recordings of membrane potential were obtained following a 30 min incubation period in either aCSF (control) media or aCSF media containing 0.05 LA. Following a 5 min baseline recording of RMP, perfusion was switched to OGD media until the neuron depolarized (anoxic depolarization; AD). (A) Representative intracellular recordings of membrane potential obtained from cortical neurons within a rat brain slice in the presence and absence of 0.05 mg/ml LA. (B) Bar graph illustrating the average latency (time to peak) of anoxic depolarization from the onset of OGD perfusion in the presence and absence of 0.05 mg/ml LA. Each data point represents mean $\pm$ SEM ($n = 3$) and (*) indicates significant difference ($p < 0.05$) between groups.

the LA-induced neuroprotection. First, we examined the effects of LA pretreatment on the electrophysiological properties of healthy neurons. These results are presented in Fig. 2. For this experiment, slices were first perfused in aCSF without LA (baseline), then perfused with aCSF containing LA at either 0.005 mg/ml or 0.05 mg/ml for 30 min, and then back to aCSF alone for another 30 min (washout). To examine the effects of LA on resting membrane potential (RMP; Fig. 2A), cells were recorded in current clamp mode and their RMP was monitored at several time points throughout the experiment. When LA was bath applied at 0.005 mg/ml, RMP increased slightly from -68 ± 2 mV to -65 ± 3 mV and then went to -64 ± 2 mV during washout ($n = 3$; $p > 0.05$). When LA was bath applied at 0.05 mg/ml, RMP increased from -66 ± 2 mV to -63 ± 3 mV and then to -62 ± 3 mV following washout ($n = 6$; $p > 0.05$). Thus, neither concentration of LA significantly altered RMP throughout the experiment. We next examined whether or not LA altered the minimal amount of current required to trigger action potentials (AP; Fig. 2B and C) or the number of action potentials generated by the maximum current step (Fig. 2B and D). During baseline (in aCSF) the minimum amount of current required to elicit APs was similar for the 0.005 mg/ml and 0.05 mg/ml experimental groups (258 ± 23 pA and 266 ± 16 pA, respectively). Data presented

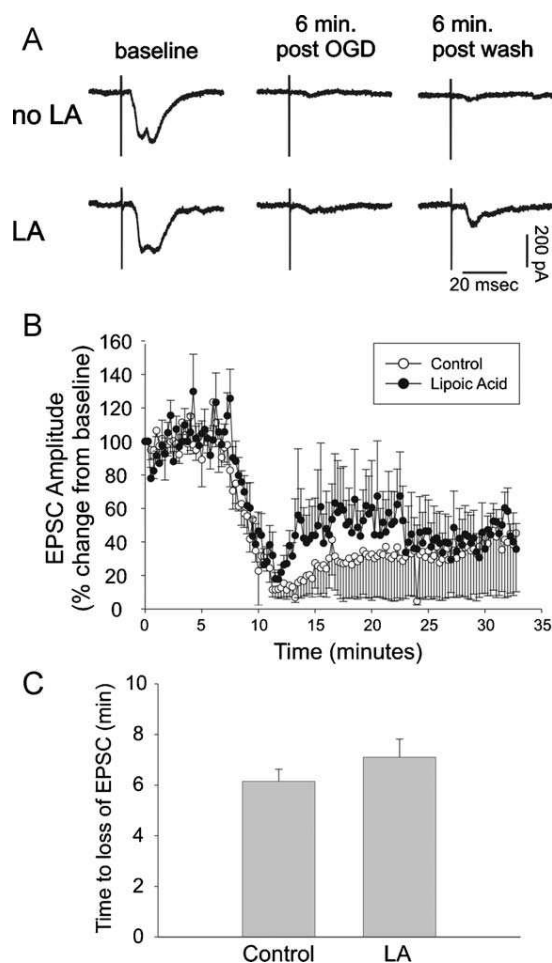


Fig. 4. Effect of LA pretreatment on the loss of EPSCs caused by OGD. Slices were preincubated for 30 min in either aCSF or aCSF + 0.05 mg/ml LA then EPSCs were evoked and recorded from the cortex. Following a 5 min of baseline recordings in aCSF (control) or aCSF + LA perfusion was switched to OGD media until EPSCs were completely lost. Then, perfusion was switched back to aCSF (or aCSF + LA) for 30 min in order to determine if the EPSCs would recover. (A) Representative recordings of EPSCs during baseline, after 6 min of OGD application, and after 5 min of washout in aCSF in the presence and absence of 0.05 LA. (B) Graphical representation of changes in EPSC amplitudes over time within OGD media in the presence and absence of 0.05 mg/ml LA ($n = 5$). (C) Bar graph illustrating the average latency to the loss of EPSC from the onset of OGD perfusion in the presence and absence of 0.05 mg/ml LA. Each data point represents mean $\pm$ SEM and (*) indicates significant difference ($p < 0.05$) between groups. Statistics were performed using one-way ANOVA.

for the remainder of the experiment are presented as percentage change from these baseline values.

Bath application of LA at 0.005 mg/ml or 0.05 mg/ml did not result in any significant change in the amount of current required to trigger APs ($-9 \pm 17\%$; $n = 3$ and $-5 \pm 11\%$; $n = 6$, respectively), or the current required during washout ($-10 \pm 13\%$ and $12 \pm 13\%$, respectively). As well, the number of action potentials elicited by the maximum current step was not significantly altered by LA or washout ($-2 \pm 27\%$ and $10 \pm 23\%$, respectively for the 0.005 mg/ml group, $n = 3$; and $-3 \pm 16\%$ and $-29 \pm 36\%$, respectively for the 0.05 mg/ml group; $n = 6$). Finally, we examined the effect of LA on synaptic transmission in healthy neurons by eliciting and measuring the amplitude of EPSCs. These data are presented in Fig. 2E and F. During baseline, the average EPSC amplitudes prior to bath application of LA at 0.005 mg/ml and 0.05 mg/ml were 95 ± 16 pA and 81 ± 35 pA, respectively. These amplitudes did not change significantly for either group throughout drug treatment or washout (96 ± 21 pA and 90 ± 11 pA, respectively for the

0.005 mg/ml group, $n = 3$; and 80 ± 24 pA and 108 ± 50 pA, respectively for the 0.05 mg/ml group, $n = 5$).

3.3. Effect of LA on electrophysiological parameters during ischemia

Following determination that LA did not affect the electrophysiological properties of neurons under normal conditions, we then determined if LA affected the electrophysiological properties of neurons under ischemic conditions *in vitro*. This was accomplished by global application of OGD media to the brain slice. We first examined the effects of LA on changes to RMP during ischemia. Cells were recorded in current clamp mode and their RMP was continuously monitored. Representative traces of membrane potential (Fig. 3A) demonstrate that following several minutes in OGD media, neurons underwent a large and sustained depolarization to approximately 0 mV (anoxic depolarization; AD; Tanaka et al., 1997; Niiyama et al., 2005). Consistent with our findings in Fig. 2A, 30 min pretreatment with LA (0.05 mg/ml) did not alter baseline RMP (from -66 ± 5 mV to -67 ± 5 mV for the LA treated group; $p > 0.05$; data not shown). Interestingly however, pretreatment with LA (0.05 mg/ml) caused a significant delay in the onset of the AD (13.37 ± 0.73 min, $n = 4$) as well as a prolonged time course to depolarization compared to the control (OGD alone) group (9.74 ± 0.36 min, $n = 3$; $p < 0.05$; Fig. 3B).

We then examined if LA could prevent the effect of ischemia on synaptic function. This was accomplished by recording EPSCs in voltage clamp mode throughout OGD perfusion in the presence and absence of LA (Fig. 4). During baseline, the average EPSC amplitudes for the control and LA-treated group were similar (194 ± 44 pA, $n = 4$ and 161 ± 48 pA, $n = 5$), respectively. EPSC amplitudes for the remainder of the experiment were then normalized to these baseline values. Once the perfusion was switched to OGD media (with or without LA at 0.05 mg/ml) EPSC amplitude for both groups rapidly decreased and followed a similar time course (6.1 ± 0.7 min for the control group and 7.1 ± 0.9 min for the LA treated group; Fig. 4B and C). Immediately after the EPSCs were lost, the perfusion was returned to aCSF media (with or without LA) in order to determine if the EPSCs would recover. Within approximately 5 min after switching from OGD to aCSF, EPSCs from slices in the control group recovered to $29 \pm 22\%$ of baseline values. In comparison, EPSCs from slices pretreated with LA (0.05 mg/ml) recovered to $60 \pm 25\%$ of baseline values. However, these values were not significantly different ($p > 0.05$; Fig. 4A).

3.4. Effect of LA on intracellular calcium

In the next series of experiments, we determined if LA causes changes in basal neuronal Ca^{2+} levels and/or changes in the glutamate-evoked Ca^{2+} response. For these particular experiments, intracellular Ca^{2+} levels were monitored in isolated neurons using the calcium-sensitive dye, fura-2 AM. Glutamate (250 μM) was used to induce an increase in intracellular calcium, which is a key component of OGD-induced excitotoxicity and ischemic stress (Lipton, 1999). For the initial experiments, LA was applied with a similar time course as during the electrophysiological experiments (30 min preincubation). This was done in order to determine if the LA-induced effects observed on AD (Fig. 3) could be mediated by alterations in intracellular Ca^{2+} dynamics. First, intracellular Ca^{2+} concentrations were measured at the beginning and the end of a 30 min preincubation period in order to determine if LA altered basal Ca^{2+} levels (Fig. 5). The results demonstrate that LA at either 0.005 mg/ml (Fig. 5A and C) or 0.05 mg/ml (Fig. 5B and D) did not cause any significant change in basal intracellular Ca^{2+} levels when compared to control ($p > 0.05$). Following the 30 min preincubation period, cells were stressed with glutamate (250 μM ; 200 s application; Fig. 5E–J). With this protocol, neither LA at 0.005 mg/ml

or 0.05 mg/ml caused any significant change in peak Ca^{2+} (Fig. 5G and H, respectively) or area under the curve (AUC; Fig. 5I and J, respectively) in response to glutamate ($p > 0.05$, respectively).

Previously, Shen et al. (2007) demonstrated that in C2C12 myotubules, 6 h pretreatment with LA was required in order to produce observable increases in intracellular Ca^{2+} levels. Therefore, for the next set of experiments, the experimental protocol was kept the same as that in Fig. 5 with the exception that the preincubation period in LA was increased to 6 h. The results are presented in Fig. 6. Six hours pretreatment with LA at either 0.005 mg/ml (Fig. 6A–D) or 0.05 mg/ml LA (Fig. 6E–H) did not result in any significant change in basal Ca^{2+} levels (Fig. 6B and F), peak Ca^{2+} response or AUC to 250 μM glutamate (Fig. 6C, G, D and H; $p > 0.05$, respectively). In comparison, pretreatment with 0.5 mg/ml LA (Fig. 6I–L) significantly increased basal Ca^{2+} levels (Fig. 6J; $440 \pm 20\%$; $p < 0.05$; $n = 3$). As well, although 0.5 mg/ml LA did not alter the peak Ca^{2+} response to glutamate (Fig. 6K), it did significantly reduce the AUC by $29 \pm 9\%$ (Fig. 6L; $p < 0.05$; $n = 3$).

4. Discussion

In this study, our results seem to suggest a potential role for calcium in mediating the lipoic acid-induced neuroprotection following stroke. We first demonstrated that in a rat model of permanent focal ischemia, acute application of LA (0.05 mg/ml) significantly reduced infarct volume within the short time frame of the experiment (6 h). We further demonstrate that these immediate neuroprotective effects of LA may be caused by a delay in the onset of anoxic depolarization and delayed depolarization time course and by alterations to intracellular Ca^{2+} homeostasis.

Much of the neuronal damage during stroke is caused by over excitation of the neurons (excitotoxicity; Lipton, 1999). It thus stands to reason that any intervention that decreases neuronal excitability could theoretically decrease the likelihood or delay the onset of excitotoxicity. One way in which excitability can be decreased is by hyperpolarizing the neurons (RMP is made more negative; Di Resta and Becchetti, 2010). However, in our experiments LA did not produce any measurable changes in RMP, consistent with previously reported findings (Wang and Chen, 2007). Another way by which neuronal excitability can be altered is by changing membrane ion conductances. For example, increased conductance of different potassium (K^+) channels (i.e. Ca^{2+} -activated K^+ channel) or γ -aminobutyric acid (GABA)-mediated chloride channels can affect excitability by either hyperpolarizing the membrane or by countering depolarization (Mann and Paulsen, 2007; Misonou, 2010; Misonou et al., 2004). Since we did not observe any measurable changes in the amount of current required to fire APs or in the frequency of AP firing, it is unlikely that LA provides neuroprotection by modulation of membrane conductances in healthy neurons.

The results presented here also demonstrated that LA did not alter synaptic transmission in healthy neurons. These data are consistent with our other findings that LA was not altering inhibitory responses (i.e. GABA mediated Cl^- conductances), but also would suggest that LA was not altering any excitatory (glutamatergic) responses. The latter is in contrast to findings by Wang and Chen (2007) which suggest that LA, at concentrations similar to what we used, facilitates glutamate release from presynaptic neurons. This disparity may be explained by differences in the experimental preparations that were used as we used rat brain slices and they used isolated synaptosomes. A disadvantage to using isolated cells is that they lack normal synaptic contacts and interactions with other neurons as well as with other cerebral cell types (i.e. glial cells) which may alter synaptic biophysics (Lossi et al., 2009). With isolated cells, it is impossible to determine functional responses to

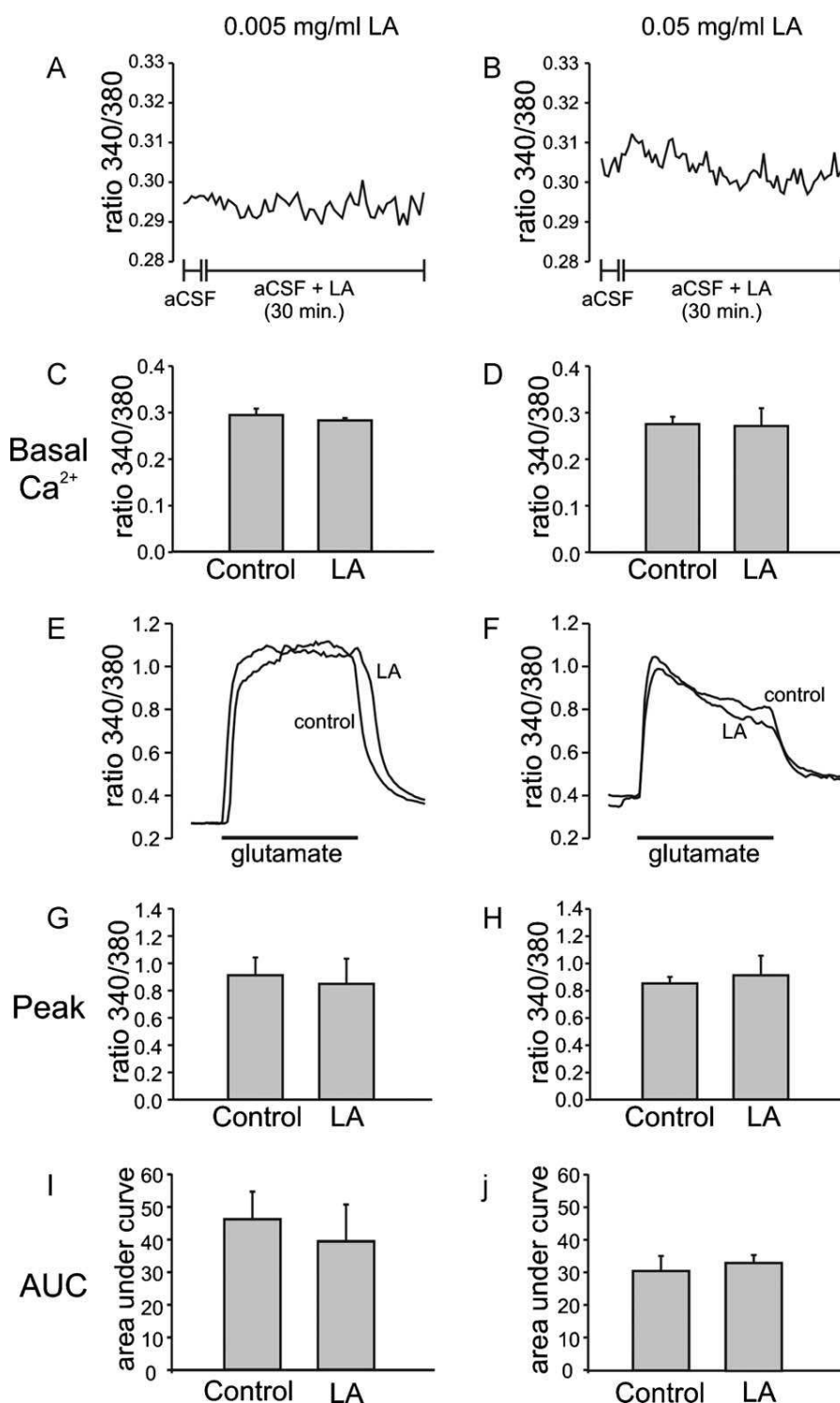


Fig. 5. Effect of 30 min pretreatment with LA on intracellular calcium levels. (A and B) Representative traces of neuronal Ca^{2+} levels during 30 min pretreatment with LA at 0.005 mg/ml (A) and 0.05 mg/ml (B). Ca^{2+} levels were recorded every 30 s. Each trace represents the average Ca^{2+} response from 10 to 20 neurons within the field of view during each experiment. (C–D) Graphical representation of the average resting neuronal Ca^{2+} levels before and after 30 min pretreatment with LA at 0.005 mg/ml (C) and 0.05 mg/ml (D). (E and F) Representative traces of neuronal Ca^{2+} responses to 250 μM glutamate application following a 30 min pretreatment with either LA (E = 0.005 mg/ml; F = 0.05 mg/ml) or control media. Following a 45 s baseline recording, glutamate was applied for 200 s, and then was washed out for another 120 s. Each trace represents the average Ca^{2+} response from 10 to 20 neurons within the field of view during each experiment. (G and H) Graphical representation of the average peak Ca^{2+} response to the application of 250 μM glutamate following 30 min pretreatment with LA (G = 0.005 mg/ml; H = 0.05 mg/ml) or control media. (I and J) Graphical representation of the average areas under the curves (AUC) during 250 μM glutamate application following 30 min pretreatment with LA (I = 0.005 mg/ml; J = 0.05 mg/ml) or control media. Prior to calculating AUC the responses were normalized to the baseline levels. Each data point represents mean $\pm$ SEM ($n = 3$).

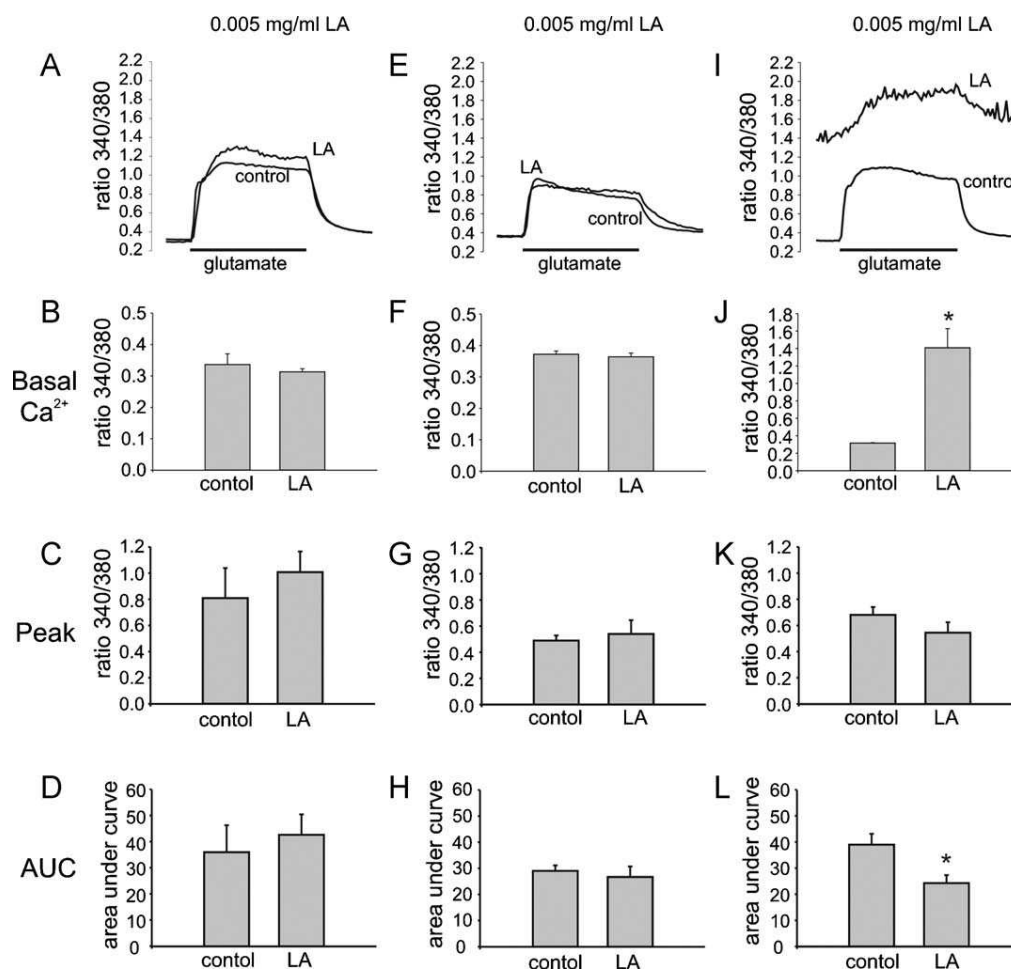


Fig. 6. Effect of 6 h pretreatment with LA on intracellular calcium levels. (A, E, I) Representative traces of neuronal Ca^{2+} responses following 6 h pretreatment with either LA (A = 0.005 mg/ml; E = 0.05 mg/ml; I = 0.5 mg/ml) or control media. Following a 45 s baseline recording, 250 μM glutamate was applied for 200 s, then was washed out for another 120 s. Each trace represents the average Ca^{2+} response from 10 to 20 neurons within the field of view during each experiment. (B, F, J) Average resting neuronal Ca^{2+} levels following 6 h pretreatment with LA (B = 0.005 mg/ml; F = 0.05 mg/ml; J = 0.5 mg/ml) or control media. These numbers were obtained by averaging the Ca^{2+} recordings within the first 45 s of the experiment. (C, G, K) Average peak Ca^{2+} response to the application of 250 μM glutamate following 6 h pretreatment with LA (C = 0.005 mg/ml; G = 0.05 mg/ml; K = 0.5 mg/ml) or control media. (D, H, L) Average areas under the curves (AUC) during 250 μM glutamate application following 6 h pretreatment with LA (D = 0.005 mg/ml; H = 0.05 mg/ml; L = 0.5 mg/ml) or control media. Prior to calculating AUC the responses were normalized to the baseline levels. Each data point represents mean $\pm$ SEM ($n = 3$) and (*) indicates significant difference ($p < 0.05$) between treatment groups.

neuronal changes. For example, [Tang and Aizenman \(1993\)](#) showed that LA can interact with the redox modulatory site of the NMDA receptor to reduce glutamate induced Ca^{2+} influx and excitability. Therefore, even if LA does cause an increase in presynaptic glutamate release, this effect may be completely counteracted post-synaptically; a functional consequence only measurable in intact, or semi-intact (i.e. slices) tissue as in our study.

Once it was determined that LA did not measurably alter any electrophysiological parameters in healthy neurons, the next step was to determine if it altered any parameters during an ischemic insult, achieved following OGD perfusion. Previous studies have demonstrated that within a few minutes after the onset of ischemia, neurons undergo a rapid depolarization (anoxic depolarization; AD) coinciding with metabolic arrest and loss of membrane integrity ([Tanaka et al., 1997](#); [Niiyama et al., 2005](#)). Consistent with previous studies, all of the neurons underwent AD within several minutes of OGD perfusion. Interestingly however, when slices were pretreated for 30 min with LA, the onset of this AD was significantly delayed. Owing to the presence of two thiol groups, dihydrolipoic acid, a metabolite of LA, is a powerful chelating agent of heavy metals ([Sigel et al., 1978](#)). [Dietz et al. \(2008\)](#) demonstrated that during the early stages of ischemia, cytosolic levels of Zn^{2+} measurably

increase. Further, they showed that this Zn^{2+} accumulation played a critical role in mitochondrial depolarization and subsequently AD and spreading depression ([Dietz et al., 2008](#)). It therefore stands to reason that Zn^{2+} chelation is a possible mechanism by which LA delays AD and provides neuroprotection.

Another possible explanation involves Ca^{2+} dysregulation during the early stages of ischemia. It is well documented that much of the neuronal damage caused by ischemia is caused by large increases in intracellular Ca^{2+} concentration ([Lipton, 1999](#)). Paradoxically, it has also been shown that modest increases in basal Ca^{2+} levels in neurons may contribute to ischemic preconditioning which provides protection against subsequent, larger ischemic insults ([Tauskela and Morley, 2004](#); [Marini et al., 2007](#)). Hence, it stands to reason that an intervention which either prevents or results in a mild increase in intracellular Ca^{2+} response to an insult may provide neuroprotection. Our results showed that following 30 min pretreatment with LA, neither basal Ca^{2+} levels nor the Ca^{2+} response to glutamate were affected. This is consistent with findings by [Shen et al. \(2007\)](#) which showed that, in myotubules, short preincubation periods did not alter Ca^{2+} levels. Because these experiments used similar preincubation times as in the electrophysiology experiments, these findings suggest that changes to

intracellular Ca^{2+} levels could not have been responsible for the shift in latency of AD. The study by [Shen et al. \(2007\)](#) also showed that longer LA preincubation times resulted in increases in intracellular Ca^{2+} levels. Based on this, we then increased our preincubation time to 6 h. However, with the two initial LA concentrations that were used, no change in calcium response was observed. Interestingly, once LA concentration was increased to 5 mg/ml, large effects on intracellular Ca^{2+} levels were recorded. First, basal Ca^{2+} levels were significantly increased. This could be explained by an increase in presynaptic glutamate release by LA, as described by [Wang and Chen \(2007\)](#). Second, the amplitude of the Ca^{2+} response to glutamate was significantly attenuated. A possible explanation for this response is that the increase in basal Ca^{2+} levels provided a preconditioning stimulus ([Tauskela and Morley, 2004; Marini et al., 2007](#)) that protects against the larger insult.

5. Conclusions

In conclusion, this study provides insight into a possible role for calcium in mediating the neuroprotective properties of lipoic acid. Taken together with the abundant literature available on the effects of lipoic acid as a potent free radical scavenger during and following ischemia, our data provide further insight into the cellular mechanism underlying the neuroprotective effects of lipoic acid. Enhanced comprehension and understanding of these mechanisms are crucial for consideration of potential therapeutic uses of LA in the management and treatment of stroke.

Acknowledgments

The authors wish to acknowledge funding support from the Atlantic Canada Opportunity Agency-Atlantic Innovation Fund (189266).

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