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

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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 Krieglstein, 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 thiobutabarbital (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 $37 \pm 1\,^{\circ}C$ using a Physiotemp feedback temperature control system (Physitemp.Instuments; 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 (Isoflo™; Abbott Laboratories, Saint-Laurent, PQ) and then decapitated. Brains were rapidly removed and immersed in ice-cold (2–3 °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, osmolarity of 295–305 mOs mol/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 submersed 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 $35 \pm 1\,^{\circ}C$ 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.

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