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Regulation of ischemic cell death by the lipoic acid–palladium complex, Poly MVA, in gerbils

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Abstract

Modulation of ischemic cell death can be accomplished via a multitude of mechanisms, such as quenching radical species, providing alternative energy sources, or altering glutamate excitation. Transient cerebral ischemia will induce apoptotic cell death selectively to hippocampal cornus ammon's field 1 of the hippocampus (CA1) pyramidal cells, while neighboring CA3 and dentate neurons are spared. Poly MVA is a dietary supplement based on the nontoxic chemotherapeutic lipoic acid–palladium complex (LAPd). LAPd is a liquid crystal that works in cancer cells by transferring excess electrons from membrane fatty acids to DNA via the mitochondria. Therefore, by its structural nature and action as a redox shuttle, it can both quench radicals as well as provide energy to the mitochondria. To understand the role of LAPd in regulating ischemic cell death, we studied Poly MVA. Male Mongolian gerbils were subjected to 5 min of bilateral carotid artery occlusion under a controlled temperature environment (37.0–38.0 °C). Animals were injected with physiological saline or either 30, 50, or 70 mg/kg of Poly MVA every 24 h beginning immediately after the occlusion until being sacrificed on experimental day 4. Damage was evaluated by analyzing nesting behavior and conducting blinded measures of viable CA1 lengths. All Poly MVA treatment dosages significantly ($p < 0.05$) reduced hippocampal CA1 damage by 72 h. Nesting scores were significantly improved after 30 and 50 mg/kg treatment but not 70 mg/kg. While nesting is usually a very accurate indicator of morphological damage, the 70 mg/kg-treated animals demonstrated excessive energy, thus ignoring the nesting material. While numerous routes offer varying degrees of CA1 neuronal survival after transient global ischemia, only the LAPd complex, which quenches radicals and provides energy to stabilize the mitochondria, offers such significant protection. Thus, the administration of Poly MVA may be a potent neuroprotective agent for victims of transient ischemic attack (TIA), cardiac arrest, anesthetic accidents, or drowning.

Keywords: Poly MVA; DNA Reductase; Hippocampus; CA1; Transient ischemia; Gerbil

Introduction

Transient global ischemia results in selective neuronal damage to hippocampal cornus ammon's field 1 of the hippocampus (CA1) neurons. While ischemic hippocampal pyramidal cells appear normal initially after transient forebrain ischemia, they die following a definite delay (Ito et al., 1975; Kirino, 1982; Kirino and Sano, 1984; Morse and Davis, 1990). This delay period of 2–4 days has been the focus of intense research in an attempt to understand the mechanism underlying ischemic neuronal death. By utilizing lipoic acid–palladium complexes (LAPd), which functions by modulating cellular energy, we wish to protect these cells from death.

Selective death of CA1 pyramidal cells follows an apoptotic cascade of events. While there is no early DNA fragmentation, as seen during classic apoptosis of thymocytes and lymphocytes (Wyllie, 1997), fragmentation is observed just before cell death. It has been detected via both dUTP-biotin nick-end labeling (TUNEL) staining and gel electrophoresis laddering (Davis et al., 1994). We have previously demonstrated an early activation of the pro-apoptotic protein, bax (Antonawich et al., 1996; Krajewski et al., 1995). This results in a shift in the dimerization ratio

Abbreviations: LAPd, lipoic acid–palladium complex; CA1, cornus ammon's field 1 of the hippocampus; TIA, transient ischemic attack.

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between the anti-apoptotic protein bcl-xl and bax (Antonawich et al., 1998). This disproportionate increase of bax results in the formation of pores in the mitochondrial membrane, as evidenced by a disruption of the mitochondrial permeability transition. These pores facilitate the passage of the electron transport chain protein, cytochrome *c*, into the cytosol, along with the release of pro-caspase 9 (Antonawich, 1999; Schendel et al., 1998). Caspase 9 is an initiator caspase that aids in the activation of the caspase family of cysteine proteases that act as terminal enzymes to destroy the cell (Zhivotovsky et al., 1998).

The LAPd molecule was originally designed as a nontoxic chemotherapeutic agent. It exists in a prescription version called DNA Reductase and a dietary supplement called Poly MVA. The active ingredient is the palladium–lipoic acid polymer, which exists as a trimer of palladium–lipoic acid joined to thiamine (Garnett, 1995). This arrangement is unique in that it allows the molecule to be both water and lipid soluble, as well as exist as a liquid crystal. This liquid crystalline structure allows it to store a great deal of energy and thus serve as a semiconductor (Garnett and Garnett, 1996). LAPd is believed to act at complex I in the mitochondria at the pyruvic dehydrogenase complex. It oscillates between an oxidized and reduced form as it accepts unpaired electrons and donates them to its enzymatic site. In cancer cells, it has been demonstrated to be an oxidizer of membrane fatty acids, serving as a redox shunt to provide a continuous river of reducing equivalents to protect DNA against oxidation (Garnett, 2002a). At the same time, the oxidation of the membrane produces membrane blistering in anaerobic conditions (blebbing) (Garnett, 2002b). Toxicology studies show no accumulation in or damage to any tissues, and the LD50 in mice is greater than 5000 mg/kg (the highest dosage tested) (Pharmakon USA, 1995a,c). The Ames tests confirm that the complex is free of mutagenicity (Pharmakon USA, 1995b).

Because both the presence of free radical scavengers (El-Abhar et al., 2002; Yamamoto et al., 1998) and the addition of an alternative energy source (Phillis and O'Regan, 2002; Sapolsky, 1986) offer limited protection from global ischemia, it was believed that this unique molecule may be a potent protector of CA1 hippocampal neurons. While alpha-lipoic acid has been demonstrated to offer some protection from ischemic damage via its antioxidant role (Clark et al., 2001; Panigrahi et al., 1996; Piotrowski et al., 2001; Wolz and Krieglstein, 1996), the LAPd complex does not contain any free lipoic acid, but rather it is uniquely bound in the aforementioned liquid crystalline structure. Because LAPd will grab excess electrons, such as free radicals, and shuttles them to complex 1 of the mitochondria (providing energy to the depleted mitochondria), it was suspected that this may be of significant benefit to aerobic systems exposed to a transient ischemic event. To determine if LAPd provided a neuroprotective effect, we administered Poly MVA (LAPd dietary supplement) to gerbils subjected to transient ischemia by 5 min of temporary bilateral carotid occlusion, then examined the animals behaviorally and morphologically.

Experimental procedures

Animals

Adult male Mongolian gerbils (Charles River Inc., NY) weighing between 45 and 60 g were used. They were housed individually, under a normal light cycle (12-h L/D), at a temperature of 20–22 °C, with food (Purina Rodent Chow) and water available ad libitum. The gerbils were randomly distributed into either the control or treatment group and sacrificed at 3 days (72 h) after transient ischemia surgery ($n = 6$ per surgical group; $n = 6$ per sham group, each experiment conducted in triplicate).

Surgical procedure

Surgical procedures were performed under halothane gas anesthesia. Gerbils were placed in an anesthetic container for 2–3 min containing 3% halothane, 70% nitrous oxide, and 27% oxygen. During the surgical procedure, the anesthetic plane was maintained using 1.5% halothane anesthesia administered through a nose cone. The carotid arteries were exposed via a midline neck incision. After the carotids were isolated, they were occluded for exactly 5 min using micro-aneurysm clips. Throughout the occlusion period, halothane was reduced to 0%. Body temperature was monitored using both a rectal and a temporalis muscle probe and maintained between 37.0 °C and 39.0 °C. At the completion of the occlusion period, the clips were removed and reperfusion was visually confirmed before the neck was closed with surgical wound clips (Kirino, 1982). Sham surgeries were conducted in a similar manner except that the carotid arteries were not occluded.

Treatment

A dose response analysis was conducted on the LAPd compound Poly MVA (Garnett McKeen Laboratory, Inc.). Poly MVA is a liquid dietary supplement that contains 23.5 mg/ml of LAPd as well as minerals (Molybdenum, Rhodium, Ruthenium), vitamins (B1, B2, B12), and amino acids (*N*-Acetyl Cysteine and Formyl Methionine). It was administered intraperitoneally (IP) immediately after ischemia, then once daily for 3 days at which time the animals were euthanized. Animals received either physiological saline or either 30, 50, or 70 mg/kg of Poly MVA (sterile filtered using a 0.2 μm Gelman filter). Previous studies have demonstrated that the Poly MVA vehicle, devoid of LAPd, is not significantly different from saline treatment (Antonawich, 2003).

Nesting behavior

One hour following ischemic surgery, each animal was exposed to novel nesting material. Two sheets of two-ply paper towel were torn in 3/4 in. strips and placed on top of the bedding material in the center of the cage. The nesting behavior of each animal (control and Poly MVA treated) was recorded every 24 h for the duration of the experiment according to our previous publication (Antonawich et al., 1997). The behavior was scored qualitatively using the following parameters: the animal received a score of "1" if it merely pulled the nesting material over to a corner. A score of "2" was awarded if the animal both pulled the material to a corner and piled it for its nest. Finally, a "3" was given if the animal pulled the material to a corner, piled, and shred it into a nest formation.

Perfusion

The animals were sacrificed with an overdose of sodium pentobarbital (0.1 ml 65 mg/ml). They were then perfused transcardially with 25–50 ml heparinized physiological saline, followed by 75–100 ml 4% paraformaldehyde (0.1 M PO_4 buffer, pH 7.2). The brain was then removed, blocked, and postfixed at 4°C for 24 h.

Histology

Coronal sections (50 μm) were cut with a vibratome beginning at the rostral hippocampus and extending caudally to the hippocampal flexure. They were mounted on gelatin-coated slides, air-dried overnight, and stained with cresyl violet. Sections chosen to be at the same level were selected from each animal. To obtain a quantitative assessment of CA1 damage, the length of viable CA1 was measured in μm. Cells counted in both hippocampal formations (right and left) were treated independently. To account for any variations in the length of the hippocampal formations measured, the total CA1 length from the subiculum to CA3 was standardized to the mean length of all samples measured. Images were captured using a Nikon Coolpix 4500 attached to a Leitz Dialux II microscope (6×). Lengths of the CA1 hippocampal region were measured with the Scion Image analysis system.

Statistics

The quantitative measurements of hippocampal damage were analyzed with repeated measures analysis of variance with side as the within-group variable and treatment as the between-group measurement. Differences between the individual treatment groups were estimated with Duncan's multiple range test. Nesting was measured using the ordinal scale system described above. Comparisons of nesting scores were carried out using ANOVA. Statistical analysis was carried out using the Statistical Analysis System (SAS Institute, Cary, NC).

Results

Nesting after 5 min of ischemia

Traditionally, the behavioral period following hippocampal damage, lesion or ischemia, has been classified as a period of locomotor hyperactivity. Ischemic gerbils also suffer deficits that result in delays in habituation and spatial mapping upon exposure to a novel testing environment. While the animals may initially demonstrate habituation deficits, they eventually compensate for these deficits (Antonawich et al., 1997). Therefore, transient global ischemia attenuates the stereotypical nest-building behavior of the gerbil, however nests are eventually made.

Five minutes of carotid artery occlusion was sufficient to hinder nesting for approximately 3 days. By 24 h after carotid occlusion, both 30 and 50 mg/kg dosages of Poly MVA exhibited significant improvements in nesting behavior ($p < 0.05$) compared to saline control-treated animals (Fig. 1). 50 mg/kg was 300% behaviorally more effective than saline or any other dose tested at 24 h after ischemia. The statistically significant improvement of the 50 mg/kg of Poly MVA was still maintained at 48 and 72 h after ischemia. 30 mg/kg was also significantly better than saline at 72 h after ischemia. However, at 48 h while 30 mg/kg still demonstrated a trend toward behaviorally improvement, we did not obtain statistical significance, most probably due to some variability in nesting scores for the Poly 30 treatment animals and resultant larger standard error values. 70 mg/kg Poly MVA-treated animals did not have improved nesting scores compared to saline. These results may be an anomaly because the animals were very hyperactive, even climbing on the bottom of the wire cage top, a behavior not typically observed with gerbils.

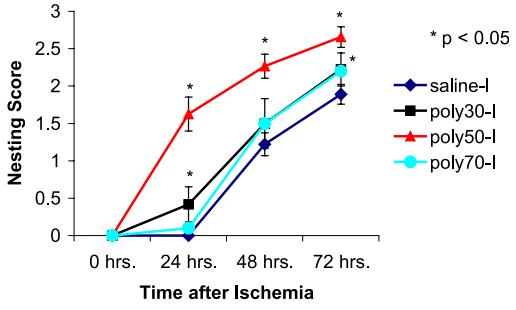

Fig. 1. Nesting behavior is disrupted in gerbils after transient ischemic damage. Treatment with Poly MVA significantly improves nesting following treatment with 50 mg/kg every 24 h ($P < 0.05$) and 30 mg/kg per 24 h at 24 and 72 h after ischemia. There were no significant differences after the 70 mg/kg per 24 h treatment ($n = 6$ per group, each experiment was conducted in triplicate).

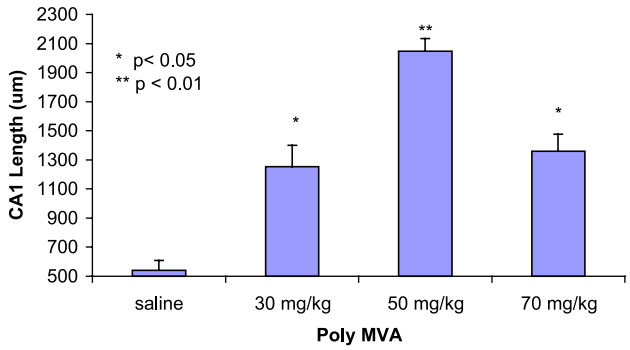

Fig. 2. CA1 protection after Poly MVA treatment. The lipoic acid–palladium-based dietary supplement Poly MVA significantly protected CA1 hippocampal pyramidal cells from transient global ischemia at 30 ($p < 0.05$), 50 ($p < 0.01$), and 70 ($p < 0.05$) mg/kg per 24 h ($n = 6$ per group, each experiment was conducted in triplicate).

Morphology

While nesting behavioral effects are apparent by 24 h, neuronal damage is not seen until 72 h after surgery (experimental day 4). The quantitative assessment of hippocampal CA1 cell death following 5 min of transient ischemia was consistent with our prior experiments. Sham surgery and treatment with the experimental dosages resulted in no significant changes (data not shown). Selective CA1 damage was typified by the appearance of pyknotic neurons beginning medially at the subiculum and continuing laterally to the larger CA3 hippocampal neurons. Poly MVA significantly (30 mg/kg per 24 h, $p < 0.05$; 50 mg/kg per 24 h, $p < 0.01$; 70 mg/kg per 24 h, $p < 0.05$) protected hippocampal pyramidal cells 72 h after carotid occlusion at every dosage tested, as evident by the length of surviving CA1 neurons (Fig. 2). Every animal treated with 50 mg/kg per 24 h responded with protection with a 2,045 μm mean length of viable CA1 neurons, which was approximately 82% of the measured region. Approximately 50% of the CA1 region was protected using either 30 or 70 mg/kg per 24 h of treatment. We expected 70 mg/kg to demonstrate similar protection to 50 mg/kg because no detrimental effect is associated with sham treatment of 70 mg/kg per 24 h; however, we did have one animal in that group experience a unilateral infarct and two others experience delays in confirmation of reperfusion. Still, if these hemispheres were removed from the experimental group, it would not approach the 80% protection observed in the 50 mg/kg-treated group.

Discussion

Ischemic cell death occurs in both focal and transient cerebral ischemia. In focal ischemia, the central area of the ischemic insult undergoes necrotic cell death, characterized by mitochondrial swelling, breakdown of plasma and nuclear membranes, and cellular lysis. The region surrounding this area, called the penumbra, shows evidence of selective cell death. However, this selective cell death has been most intensively studied in the hippocampal formation. Hippocampal CA1 neurons die after a brief episode of transient forebrain ischemia, while adjacent CA3 neurons do not. Furthermore, the CA1 neurons appear to function in a grossly normal fashion after a brief ischemic exposure, but ultimately show a variety of behaviors that culminate in cell death between 2 and 4 days after ischemia (Davis and Antonawich, 1997).

The penumbral and selective hippocampal CA1 cells are believed to die via the apoptotic cell death cascade. In this study, we utilized the bilateral carotid artery occlusion model of transient cerebral ischemia in the gerbil to demonstrate the neuroprotective effects of the lipoic acid–palladium complex, dietary supplement, Poly MVA. LAPd complexes are being tested as effective nontoxic anti-neoplastic agents. Electrochemistry studies have suggested that the anticancer effects work via shuttling of membrane fatty acid electrons to DNA via the mitochondria.

The donation of electrons to the mitochondria could be of particular interest in terms of the ischemic hippocampus. Not only is no energy available during an interruption in blood flow, but also upon reperfusion there is a stress-induced increase in serum glucocorticoids, which exacerbates damage by disrupting glucose utilization (Davis and Antonawich, 1997; Davis and Morse, 1991; Morse and Davis, 1990; Phillis and O'Regan, 2002). Administration of alternative brain fuels such as mannose, fructose, or the ketone beta-hydroxybutyrate has been shown to reduce some of the hippocampal damage (El-Abhar et al., 2002; Sapolsky, 1986). By maintaining the electron flow down the mitochondrial chain, via LAPd donation, the integrity of the chain may be maintained.

An interruption in blood flow will also lead to the generation of free radicals because oxygen is no longer present as the final electron acceptor. Furthermore, reperfusion will result in the production of radical oxygen species (hydroxyl radical, superoxide anion, and hydrogen peroxide) from numerous sources (xanthine oxidase, arachidonic acid metabolism, catecholamine oxidation, amine oxidase activity, mitochondrial leakage, and activated white blood cells) (Hall, 1993; Hall et al., 2000; Liu et al., 1998; Panigrahi et al., 1996). Because LAPd is a polymer that exists as a liquid crystal it has a large redox potential, making it a potent antioxidant agent. Therefore, the use of Poly MVA (LAPd) will combat the pathological effects of the ischemic condition at multiple sites of action.

The typical adult Mongolian gerbil will build a nest within 12–24 h following novel exposure to nesting material. The gerbil does this in a species-specific manner by grasping paper or straw between its forepaws, pulling it into a pile, then proceeding to chew and shred the material. Our prior studies have established the reliability of a disruption

(or delay) in nesting behavior to the morphological damage incurred to the CA1 region of the hippocampus (Antonawich et al., 1997). Poly MVA significantly improved nesting behavior following treatment with both 30 and 50 mg/kg per 24 h of Poly MVA (Fig. 1). However, 70 mg/kg animals did not demonstrate any differences in nesting behavior compared to saline treatment. However, these animals were extremely energetic, exhibiting activities not ordinarily observed in gerbils. Gerbils (60 g) frequently jumped to the wire cage tops to climb, however, no overaggressive behavior was observed in these animals. These effects were believed to be associated with the excess energy afforded by the Poly MVA; however, we believe this distracted the animals from their innate behavioral task of nesting because the lack of nesting did not associate with morphological damage. To the contrary, 70 mg/kg protected approximately 54% of the CA1 length.

All of the animals tested demonstrated some beneficial morphological effect following Poly MVA treatment (Fig. 2). 50 mg/kg per 24 h offered a significant protection ($p < 0.01$) compared to saline treatment with almost 80% protection of the CA1 length. Poly MVA appears to offer such dramatic benefits due to its multiple therapeutic sites of action. It not only quenches radicals, but also maintains mitochondrial effectiveness via its electron transfer to complex I, thereby stabilizing mitochondrial integrity. Therefore, cancer cells that switch to a hypoxic metabolism, and therefore rely on anaerobic respiration (Das, 2002; Hockel and Vaupel, 2001; Semenza et al., 2001; Subarsky and Hill, 2003), will be subjected to toxic effects of the LAPd via the mitochondria. Cells dependent on aerobic respiration, subjected to ischemia or hypoxia, will be protected from damage. Future studies plan on focusing on both the delayed as well as prophalatic administration of the LAPd complexes.

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