Anti-Oxidative Effect of Lipoic Acid in Spinal Cord Ischemia/Reperfusion

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Key Words
Ischemia/reperfusion · Lipoic acid · Spine

Abstract
Objective: Lipoic acid (LA) is an effective anti-oxidant agent that can scavenge free radicals in biological systems. The aim of this research was to study the probable protective effect of LA in spinal ischemic/reperfusion (I/R) injury. Materials and Methods: Thirty male Wistar rats, weighing 230–285 g, were assigned randomly into 3 groups (10 animals in each group): sham spinal I/R, and spinal I/R + LA. The spinal I/R + LA rats received LA 100 mg/kg subcutaneously 3 days prior to ischemia induction and 3 days after. The induction of ischemia lasted for 30 min. Results: At 72 h postoperatively, the neurological status was worse in the I/R group than the sham group (p < 0.05). The neurological status of animals in the LA-treated group appeared better than the I/R group (p < 0.05). In the I/R group, tissue glutathione peroxidase (GPx) and super oxide dismutase (SOD) activity were significantly less compared to the control group (p < 0.05). In the LA-treated group, tissue GPx and SOD levels were higher compared to the I/R group (p < 0.05). Conclusions: LA pretreatment reduced neurologic injury in the rats, most probably by maintaining the oxidant/anti-oxidant ion balance during spinal cord ischemia. Reperfusion may have contributed to the protective effects seen in the LA pretreatment.

Introduction
Thoracoabdominal aortic surgery and aortic cross clamping may induce transient spinal ischemia and lead to various degrees of spinal cord injury, including paraplegia [1, 2]. This complication has been attributed to temporary or permanent ischemia of the spinal cord caused by interruption of the blood supply during aortic cross-clamping [3], dissection, rupture, and prolonged clamp times [4]. Clinical adjuncts designed to reduce ischemic times, swelling of the spinal cord and monitor neurological function postoperatively have improved outcomes but have not eliminated the problem [5, 6]. The neurological deficits resulting from ischemia and reperfusion (I/R) injury may potentially be ameliorated by pharmacological manipulation. For this reason, our interest has centered on the basic mechanisms of injury of nervous tissue caused by ischemia and reperfusion [7].
Although the exact mechanism of I/R injury is not fully understood, it is believed that oxidative stress plays a pivotal role in triggering lipid peroxidation, DNA damage and specific gene expression [8]. Oxygen-derived free radicals after reperfusion of an ischemic spinal cord may be partly responsible for neuronal destruction. Pharmacologically based measures to prevent spinal cord injury have been pursued, consisting of hypothermia, anesthetic agents, calcium channel blockers, free radical scavengers, and immune system modulation [9].

Lipoic acid (LA) (1,2-dithiolane-3-pentanoic acid), synthesized by animals and humans, is a cofactor in multiple enzyme complexes that catalyze the oxidative decarboxylation of γ-keto acids, such as pyruvate, γ-ketoglutarate and branched-chain γ-keto acids [10]. Recently, it has been shown that LA exerts powerful anti-oxidant effects, which has led to studies of the use of LA in a number of oxidative stress conditions [11]. Previous studies have shown that LA can ameliorate I/R injury in organs such as the brain [12]. In this study, we investigated the effect of LA administration on neurological injury using a rat infrarenal abdominal aortic clamping model.

Materials and Methods

Animal Groups
Thirty male Wistar rats, weighing 230–285 g, were randomly assigned to 3 groups of 10 animals per group: sham, spinal I/R, and spinal I/R + LA (100 mg/kg). Rats in spinal I/R + LA group received 100 mg/kg LA subcutaneously 3 days prior to ischemia induction and 3 days after I/R induction. An equal volume of saline was injected in the spinal I/R group.

Animal Surgery
Anesthesia was induced by an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). A midline laparotomy was performed and the viscera were pushed to the right side. Upon opening the retroperitoneum, the abdominal aorta was identified and clamped with a microvascular clamp distal to the left renal artery. Successful arterial occlusion was indicated by the immediate cessation of pulse in the distal artery and a pale appearance in the hind paws of the animals. After 30 min of aortic occlusion, the aneurysm clip was removed and the incision was closed. The sham surgery was identical to the surgical clamping procedure except the aortic aneurysm clip was removed and the incision was closed. The sham group was tested the same way as the ischemia group, but the aorta was not clamped.

Behavioral Test
Behavioral tests were performed and graded in all animals before ischemia and at day 3 after ischemia. Motor function was quantified by assessment of ambulation and placing and stepping responses. The score for ambulation, i.e., walking with lower extremities (LE), was as follows: normal (symmetric and coordinated ambulation): 0–0.25; toes flat under body when walking with, ataxia present: 1.25; knuckle-walking: 2.25; movement in LE but unable to knuckle-walk: 3.25; and no movement, drags LE: 4.25. Placing/stepping reflex was assessed by dragging the dorsum of the hind paw over the edge of a surface. This normally evoked a coordinated lifting and placing response (e.g., stepping): normal: 0.25; weak: 1.25; and no stepping: 2.25. A motor deficit index was calculated for each rat at each time interval. The final index was the sum of the scores (walking with LE and placing/stepping reflex). The maximum deficit was indicated by a score of 6. Animals with motor deficit index ≥3 were considered paraplegic, whereas animals with index <3 were considered nonparaplegic [14]. Behavioral tests were observed for 3 min.

Measurement of GPx Activity
The GPx activity was determined as described by Paglia and Valentine [15]. Briefly, after mixing 40 ml of the sample with 10 ml of t-butyl hydroperoxide and a solution of distilled water containing 10 mg glutathione, glutathione reductase, NADPH, buffer (0.25 M KH₂PO₄ and 0.025 M Na₂EDTA), and 940 μl K buffer, the GPx activity was measured at 10-second intervals for 60 s by recording the rate of light absorption. Protein levels were determined using the BCA assay kit.

Measurement of Superoxide Dismutase Activity
Superoxide dismutase activity was determined as described by Sun et al. [16]. This method depends on the inhibition of nitroblue tetrizolium reduction by xanthine-xanthine oxidase used as a superoxide generator. SOD activity was designated as a unit for mg protein of intestinal tissue. One SOD activity was expressed as the amount of enzyme that causes 50% inhibition of the rate of nitroblue tetrizolium reduction.

Determination of Lipid Peroxidation
We studied the effect of LA on lipid peroxidation, which was measured in terms of MDA. Lipid peroxidation products in the spine were determined by measuring MDA using the method of Yagi [17]. Briefly, 20 μl of the sample was placed in a glass centrifuge tube; 4.0 ml of 1/12 N H₂SO₄ was added and mixed gently; 0.5 ml of 10% phosphotungstic acid was added and mixed. After being allowed to stand at ambient temperature for 5 min, the mixture was centrifuged at 1,600 g for 10 min. The supernatant was discarded and the sediment mixed with 2.0 ml of 1/12 N H₂SO₄ followed by 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged at 1,600 g for 10 min. The sediment was suspended in 1.0 ml of distilled water, and 1.0 ml of 0.67% (w/v) TBA reagent was added. The reaction mixture was heated at 95°C for 60 min. After cooling with tap water, 5.0 ml of n-butanol was added and the mixture was shaken vigorously. After centrifugation at 1,600 g for 15 min, the n-butanol layer was taken for fluorometric measurement at 553 nm with excitation at 515 nm.

Statistical Analysis
All results are presented as mean ± standard deviation (SD). Differences between groups in the motor deficit index score and paraplegia rate were carried out using 1-way Mann-Whitney test.
Significant differences were defined as $p < 0.05$, and were determined using SPSS 13. Differences of biochemical factors among various groups were tested for statistical significance using the 1-way ANOVA test and Turkey’s post test.

Results

Neurological Outcome
All sham-operated rats had a normal postoperative neurological outcome, whereas all rats in the I/R group showed severe neurological deficits, including total paraplegia ($4.85 \pm 0.69$). At 72 h postoperatively, the neurological status was worse in the I/R group than in the sham group ($p < 0.05$) and the neurological status of animals in the LA-treated group ($3.28 \pm 0.48$) appeared better than the I/R group ($p < 0.05$). There was no neurological deficit in sham group.

Example GPx Activity
The tissue GPx activities were less in the I/R group ($0.8 \pm 0.24 \text{ U/mg protein}$) than control ($2.34 \pm 0.25 \text{ U/mg protein}$) and I/R + LA ($1.59 \pm 0.24 \text{ U/mg protein}$). The difference between IR, IR + LA and control group was statistically significant ($p < 0.05$).

SOD Activity
The tissue SOD activities were less in the I/R group ($0.81 \pm 0.19 \text{ U/mg protein}$) than control ($1.73 \pm 0.36 \text{ U/mg protein}$) and IR + LA group ($1.17 \pm 0.18 \text{ U/mg protein}$). The difference between IR, IR + LA and control group was statistically significant ($p < 0.05$).

MDA Levels
The tissue MDA levels were higher in the I/R group ($3.03 \pm 0.33 \text{ nmol/ml}$) than control ($1.52 \pm 0.35 \text{ nmol/ml}$) and IR + LA group ($2.35 \pm 0.30 \text{ nmol/ml}$). The difference between IR, IR + LA and control groups was statistically significant ($p < 0.05$).

Discussion
We have shown that 30 min ischemia and 3 days reperfusion after LA administration have a significant effect on spinal cord function, thereby demonstrating that LA administration can reduce neurologic injury in a rat model of spinal cord ischemia.

Spinal cord I/R injury is defined by a complex cascade of events, including increased liberation of reactive oxygen species from a variety of cell types. There is increasing evidence that free radicals are generated by I/R and they contribute to tissue injury [18–20]. Reactive oxygen species attack a variety of critical biological molecules, including membrane lipids, essential cellular proteins, and DNA [21]. Our results showed that LA treatment attenuated reduction of GPx and SOD activity induced by spinal I/R, and reduced MDA levels as an index of lipid peroxidation after spinal I/R.

The SOD is the first line of defense against free radical generation. It has been reported that total SOD is down-regulated following spinal cord I/R [22]. Decreased SOD renders a tissue susceptible to oxidant injury. Therefore, the elevated SOD levels induced by LA may contribute to reduction of superoxide radicals following spinal cord I/R [2], as evidenced by the enhancement of SOD and GPx activities after treatment with LA of our study, thereby suggesting that these agents modulate anti-oxidative capacity in the spinal tissue. It is possible that LA may scavenge singlet oxygen by a higher singlet oxygen quenching capacity [23], and that it may also scavenge superoxide and peroxyl ROS. It was reported that LA appears to participate in the recycling of other important biologic anti-oxidants, such as vitamins E and C, and glutathione, especially during oxidative stress [24].

Following spinal cord ischemia and during reperfusion, lipid peroxidation occurs within the cell membrane. MDA is a metabolite of lipid peroxidase and it increases after spinal cord ischemia [25]. The levels of MDA demonstrate lipid peroxidation, which is thus considered to be evidence of reperfusion injury. Our finding of an increased level of MDA in the I/R group that was higher than the I/R + LA group confirmed previous reports [26] regarding the increased level of MDA in spinal injury. Belboul et al. [27] showed an increase in MDA levels in patients who undergo coronary artery surgery following 30 min of reperfusion. After removing the clamps, MDA levels increased for a second time. Lu et al. [28] reported increased MDA levels at all times of reperfusion. After ischemia, the motor neurons of the spinal cord undergo necrosis as well as delayed neuronal death. Despite recovery of blood flow, motor neurons that initially appear to have survived the ischemic insult go on to die a few days later [26]. This phenomenon is compatible with delayed deterioration of neurologic function after spinal cord ischemia. Our study showed that LA administration improved the neurological function score significantly.
Conclusions

LA pretreatment reduced neurologic injury in a rat model of spinal cord ischemia during 30 min ischemia and 3 days reperfusion. It is likely that maintaining the oxidant/anti-oxidant ion balance during spinal cord ischemia and reperfusion may have contributed to the protective effects seen in the LA pretreatment.

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References

3. Gilling-Smith GL, Worswick L, Knight PF, Kouchoukos NT, Masetti P, Rokkas CK, Lintott P, Hafez HM, Stansby G: Spinal cord protective effects seen in the LA pretreatment. is ischemia and reperfusion may have contributed to the oxidant/anti-oxidant ion balance during spinal cord and 3 days reperfusion. It is likely that maintaining the model of spinal cord ischemia during 30 min ischemia
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