Free Rad. Res., Vol. 23, No. 4, pp. 365–370
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THE FREE RADICAL SCAVENGER, α-LIPOIC ACID, PROTECTS AGAINST CEREBRAL ISCHEMIA-REPERFUSION INJURY IN GERBILS

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(Received August 16th, 1994; in revised form, November 8th, 1994)

α-Lipoic acid (thioctic acid) was tested for its neuroprotective activity in a Mongolian gerbil model of forebrain ischemia/reperfusion. Adult gerbils were treated for 7 days with two intraperitoneal injections per day of α-lipoic acid (20 mg/kg), vehicle or saline and on the 7th day the animals were subjected to 5 min of forebrain ischemia. Ischemic injury was assessed by monitoring the increases in locomotor activity and from the extent of damage to the CA1 hippocampal pyramidal cell layer after 5 days of recovery. By both criteria, α-lipoic acid was neuroprotective against ischemia/reperfusion evoked cerebral injury.

KEY WORDS: α-lipoic acid, cerebral ischemia, free radicals, gerbils, lipid peroxidation

INTRODUCTION

Free radicals have been implicated as causative agents in ischemia-reperfusion induced injury to the central nervous system. Electron spin resonance studies have demonstrated the formation of oxygen-derived free radicals in the brain[1,2] and there is evidence of free radical induced lipid peroxidation following cerebral ischemia-reperfusion.[3–5] There is also evidence to indicate that free radical scavengers and antioxidants can protect against ischemia-reperfusion damage.[2,6–11]

α-Lipoic acid (thioctic acid) is a co-factor for mitochondrial alphaketo-acid dehydrogenases and is covalently bound as lipoamide to some enzymes in animals.[12] Together with its reduced form, dihydrolipoic acid, α-lipoic acid also participates in reactions which reduce the oxidized forms of the free radical scavengers, α-tocopherol and glutathione.[13–16] In addition α-lipoic acid possesses intrinsic free radical scavenging properties.[17] This combination of activities would be expected to endow α-lipoic acid with an ability to protect the brain against ischemia/reperfusion injury.

α-Lipoic acid has been used in the treatment of diseases in which its levels were found to be low, including polyneuritis, liver cirrhosis, atherosclerosis and diabetes mellitus. Oxygen derived free radicals and free radical mediated oxidation of lipids and proteins have been implicated in the etiology of these pathologies.[18] It was therefore of interest to determine whether α-lipoic acid could protect in vivo against ischemia-reperfusion induced injury to gerbil CA1 hippocampal neurons. The gerbil model for cerebral ischemic damage is especially appropriate for the evaluation of

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putative cerebroprotective agents. The absence of vascular connections between the forebrain and hindbrain circulations makes it possible to evoke a forebrain ischemia, without disruption of the essential cardiovascular and respiratory activities of the hindbrain. In addition to histopathological measurements of CA1 damage, neurological evidence of ischemic injury is available as a readily measurable hypermotility. Previous studies have demonstrated that the degree of hippocampal damage is positively correlated with this increase in locomotor activity.[19,20]

MATERIAL AND METHODS

Experimental Protocols

Three groups of animals were tested. (1) an ischemic control group (n = 18) which was injected with saline twice daily for 7 days prior to the ischemic episode; (2) an ischemic α-lipoic acid group (n = 14) which was administered α-lipoic acid (20 mg/kg; Calbiochem) dissolved in 0.05 ml of vehicle (25% DMSO: 75% polyoxyethylenesorbitan mono-oleate; Sigma) intraperitoneally twice daily, commencing 7 days prior to the ischemic insult, (3) an ischemic vehicle control group (n = 11) which received vehicle only on the same injection schedule.

Gerbils were weighed and evaluated for basal levels of locomotor activity prior to commencing drug administration. They were weighed and tested for locomotor activity again on the 5th day of injections prior to surgery. Locomotor activity was measured on the 7th day of injections prior to the 5 min period of ischemia and at 5 hrs, 24 hrs and 5 days post ischemia.

Techniques of Cerebral Ischemia-Reperfusion and Damage Assessment

Male Mongolian gerbils (Meriones unguiculatus, 50–55 gm wt) were obtained from Harlan Sprague-Dawley (Wilmington, Mass.). Under anesthesia induced with pentobarbital sodium (50 mg/kg), loops were placed around the common carotid arteries and exteriorized through the dorsum of the neck. After a 48 hr recovery period, the gerbils were tested for spontaneous locomotor activity in a Stoelting 31410 Modular Electronic Activity monitor. Under manual restraint, the gerbils were 'stroked' for a 5 min period by traction on the carotid artery snares, after which the loops were withdrawn from the neck. Successful occlusion of the carotid arteries was evident from a complete bilateral ptosis and the adoption by the gerbil of a 'hunched' posture with the head dropped. Successful reperfusion following withdrawal of the snares was almost immediately apparent, with the reappearance of head and neck tone, a disappearance of ptosis, and a rapid resumption of normal motor activity. All ischemic animals were maintained in a warmed (30–32°C) enclosure for 5 hr post-ischemia. Animals were then retested for locomotor activity at 5 hr, 24 hr and 5 days. On the 5th day, the gerbils were reanesthetized, and perfused with saline and fixative via a cannula in the ascending aorta. After fixation, the brains were embedded, sectioned, and stained with cresyl violet. The extent of hippocampal CA1 pyramidal cell loss was assessed 'blind' from representative sections on slides. The histopathological scoring system was based on that used and illustrated in a previous study with: 0 = no apparent cell necrosis; 1 = few single cell and/or cell group necrosis; 2 = larger areas of shrunken, necrotic cell groups or missing cells; 3 = most cells necrotic or missing; 4 = virtually complete absence of intact pyramidal cells in the CA1 area. Complete details of the technical procedures

TABLE 1
Locomotor activities of gerbils prior to, and after, 5 days of intraperitoneal injection of saline, α-lipoic acid + vehicle or vehicle administration. Each data value represents the mean ± S.E.M. for animals in that group. * $P < 0.05$ in comparison with initial rate of activity.

Group	Basal activity Pre-injection (cts/min)	Activity on 5th Injection Day (cts/min)
Saline Control n = 18	75.6 ± 8.9	68.9 ± 8.3
α-Lipoic Acid n = 14	70.8 ± 7.8	66.2 ± 9.2
Vehicle Control n = 11	80.9 ± 8.9	54.2 ± 5.0*

for measurement of locomotor activity and assessment of hippocampal CA1 damage have been described in a previous publication.[7]

Motor activity data were compared using a one-way analysis of variance and the Student's t-test. Hippocampal CA1 pyramidal cell injury scores were compared with a Mann-Whitney U-test.

RESULTS

Locomotor Activity

To evaluate the possible effects of repeated injections of saline, drug + vehicle or vehicle, locomotor activity prior to the injection sequence was compared with that at 5 days (prior to surgery) (Table I). Activity was not altered in the saline or drug-treated animals, but was significantly reduced in the gerbils receiving only the vehicle. There was no weight loss in any of the groups.

Following a 5 min period of bilateral carotid artery occlusion, the saline-treated control ischemic animals displayed characteristically elevated levels of motor activity at all three testing intervals (Table II). Although the α-lipoic acid pretreated animals were also more active than prior to ischemia, the percentage increases at 24 hr and 5 days were significantly lower than those of the ischemic controls. At 5 hrs, the percentage increase in activity in the vehicle treated gerbils was even higher than that

TABLE 2
Locomotor activities of ischemic gerbils. Initial, pre-ischemic, basal levels of activity for each group were used as controls (100%). Significance values for differences between saline treated ischemic controls and drug or vehicle treated animals * $P < 0.05$ ** $P < 0.01$. Difference between α-lipoic acid and vehicle injected animals °° $P < 0.01$, °°° $P < 0.001$.

Group	Pre-ischemic Basal Activity (%)	Activity as Percentage of Initial Basal Activity (%) 5h	24h	5 days
Saline Control n = 18	100 ± 9.8	194.6 ± 15.6	228.7 ± 17.7	185.9 ± 14.4
α-Lipoic Acid n = 14	100 ± 11.6	154.6 ± 14.0°°	148.1 ± 12.6**/°°°	126.3 ± 8.6**/°°
Vehicle Control n = 11	100 ± 11.7	312.5 ± 46.2*	247.2 ± 22.9	184.7 ± 17.6

of the ischemic controls, but this trend was not apparent at 24 hrs and 5 days. The low, pre-ischemic, level of locomotor activity of this group may have contributed to the large percentage increase in activity at 5 hrs.

Histopathology

Five days after the ischemic episode, widespread damage to the CA1 region of the hippocampus was evident in the brains of the control ischemic gerbils (Table III). Most pyramidal cells either presented a shrunken appearance with condensed nuclei and minimal cytoplasm, or had disappeared leaving a vacuole-like space. Damage to, and loss of, pyramidal cells in the CA1 region of the hippocampus was reduced in gerbils which had received α-lipoic acid, indicating that the drug had significantly ($p < 0.001$) attenuated the extent of ischemic injury. By contrast, damage to the CA1 region of the vehicle treated animals was similar to that in the ischemic controls.

DISCUSSION

Free radicals have been implicated in the tissue injury occurring during cerebral ischemia, trauma and in several neurological diseases. Free radical production has been detected in rat and gerbil brains during ischemia/reperfusion[1-4,21,22] and cerebroprotection against ischemic injury has been demonstrated following the administration of free radical scavengers and inhibitors of free radical formation.[22,23] To further evaluate the role of free radicals in cerebral ischemia/reperfusion injury, we have tested α-lipoic acid, a naturally occurring compound and potent scavenger of hydroxyl radicals[17] for cerebroprotective activity in a gerbil global forebrain ischemia model. Our results show, for the first time, that α-lipoic acid exerts cerebroprotective activity in a cerebral ischemia model, as demonstrated by the reduction in hippocampal CA1 pyramidal cell injury and attenuation of ischemia/reperfusion-induced locomotor activity.

Our results show that subchronic treatment with α-lipoic acid protects hippocampal CA1 pyramidal neurons against ischemic injury. α-Lipoic acid, and its reduced form, dihydrolipoic acid, have previously been tested for their ability to protect central neurons from excitotoxic and ischemic insults. Prehn and colleagues[24] have shown that acute administration of a single dose of dihydrolipoic acid (but not α-lipoic acid) had modest protective activity in mouse and rat middle cerebral artery occlusion focal ischemia models, reducing the area of brain infarction. However, neither α-lipoic acid nor dihydrolipoic acid, administered in a single dose of 50–100 mg/kg, was capable of reducing hippocampal CA1 neuronal damage in rats subjected to 10 mins of global ischemia elicited by bilateral common carotid occlusion combined with hypotension.

Following subchronic (10 days) administration, both α-lipoic acid and dihydrolipoic

TABLE 3

Values are means + S.E.M.. See Methods section for assessment of CA1 hippocampal pyramidal cell injury; 0 = no damage; 4 = complete loss of pyramidal cells. Significance values for α-lipoic acid treated animals in comparison with control ischemic animals calculated by Mann-Whitney U-test *** $P < 0.001$. Difference between α-lipoic acid and vehicle injected animals °° $P < 0.01$.

Condition	Injury Scores
Saline Control (n = 18)	2.9 ± 0.09
α-Lipoic Acid (n = 14)	2.2 ± 0.15***/°°
Vehicle Control (n = 11)	2.7 ± 0.20

acid protected striatal neurons against excitotoxic death mediated by N-methyl-D-aspartate receptor agonists and malonic acid.[25] After a 15 day oral pretreatment period, α-lipoic acid was observed to enhance memory in aged mice.[26]

As α-lipoic acid is readily interconvertible with dihydrolipoic acid in vivo,[27] our results do not distinguish between the relative roles of these two compounds in protecting neurons against ischemic injury. The lack of effect of acutely administered α-lipoic acid in the rodent middle cerebral artery occlusion models referred to above,[24] may suggest either that dihydrolipoic acid crosses blood-brain and cell membrane barriers more readily following acute administration than does α-lipoic acid, or alternatively that α-lipoic acid exerts much of its cerebroprotective activity as its reduced form and that subchronic administration is required to allow time for this conversion. Either explanation could account for the lack of cerebroprotective activity of acutely administered α-lipoic acid in the rodent middle cerebral artery occlusion experiments[24] and for the failure of topically applied α-lipoic acid, but not of dihydrolipoic acid, to protect neurons in primary cultures against glutamate neurotoxicity.[19]

If, as the evidence indicates, lipoic acid exerts cerebroprotective activity against ischemic injury, it is likely that free radical scavenging is involved. A recent, comprehensive, evaluation of the antioxidant properties of α-lipoic acid and dihydrolipoic acid[28] demonstrated that lipoic acid is a powerful scavenger of hydroxyl radicals and can inhibit iron-dependent ·OH generation and ox-brain lipid peroxidation. In contrast, dihydrolipoic acid accelerated iron-dependent ·OH generation and lipid peroxidation. Both compounds were powerful scavengers of hypochlorous acid. The observation that dihydrolipoic acid can exert pro-oxidant effects may indicate a need for caution in its use as an antioxidant. In the hands of other investigators, however, dihydrolipoic acid has appeared to be a potent inhibitor of ·OH and O_2^- induced cellular damage.[17,20] Furthermore, if α-lipoic acid and dihydrolipoic acid increase the levels of reduced glutathione and α-tocopherol in neurons,[13–16] the capacity of neurons to scavenge free radicals would be enhanced.

In conclusion, these results demonstrate for the first time that α-lipoic acid administration results in a significant degree of cerebroprotection following a global forebrain ischemia. This finding suggests a potential role for this compound as a dietary supplement in the prophylaxis and treatment of acute and chronic neurological disorders and provides further evidence for an involvement of free radicals in cerebral ischemic injury.

Acknowledgements

Support by PHS grant NS 26912-06 and a grant-in-aid from the American Heart Association.

References

1. I. Zini, A. Tomasi, R. Grimaldi, V. Vannini and L.F. Agnati (1992) Detection of free radicals during brain ischemia and reperfusion by spin trapping and microdialysis, *Neuroscience Letters*, **138,** 279–282.
2. S. Sen and J.W. Phillis (1993) Alpha-phenyl-tert-butyl-nitrone (PBN) attenuates hydroxyl radical production during ischemia/reperfusion injury of rat brain: an EPR study. *Free Radical Research Communications*, **19,** 255–265.
3. R.A. Floyd (1990) Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB Journal*, **4,** 2587–2597.
4. E.D. Hall and J.M. Braughler (1989) Central nervous system trauma and stroke, II. Physiological and pharmacological evidence for involvement of oxygen radicals and lipid peroxidation, *Free Radical Biology and Medicine*, **6,** 303–313.
5. Y. Kinuta, H. Kikuchi, M. Ishikawa, M. Kimura and Y. Itokawa (1989) Lipid peroxidation in focal cerebral ischemia, *Journal of Neurosurgery*, **71,** 421–429.

6. O. Uyama, T. Matsuyama, H. Michishita, H. Nakamura and M. Sugita (1992) Protective effects of human recombinant superoxide dismutase in transient ischemic injury of CA1 neurons in gerbils, *Stroke*, **23,** 75–81.
7. C. Clough-Helfman and J.W. Phillis (1991) The free radical trapping agent N-tert-butyl-α-phenylnitrone (PBN) attenuates cerebral ischemic injury in gerbils, *Free Radical Research Communications*, **15,** 177–186.
8. Y. Ikeda and D.M. Long (1990) The molecular basis of brain injury and brain edema: the role of oxygen free radicals, *Neurosurgery*, **27,** 1–11.
9. B. Halliwell (1992) Reactive oxygen species and the central nervous system, *Journal of Neurochemistry*, **59,** 1609–1623.
10. J.W. Schmidley (1990) Free radicals in central nervous system ischemia, *Stroke*, **21,** 1086–1090.
11. J.W. Phillis (1989) Oxypurinol attenuates ischemia-induced hippocampal damage in the gerbil, *Brain Research Bulletin*, **23,** 467–470.
12. P.C. Jocelyn (1972) *Thiols and disulphides in animal tissues, Biochemistry of the SH groups*, Academic Press, London, 1.
13. A. Bast and G.R.M.M. Haenen (1988) Interplay between lipoic acid and glutathione in the protection against microsomal lipid peroxidation, *Biochimica et biophysica Acta*, **963,** 558–561.
14. E. Busse, G. Zimmer, B. Schopol and B. Kornhuber (1992) Influence of α-lipoic acid in intracellular glutathione in vitro and in vivo, *Arzneim-Forschung/Drug Research*, **42,** 829–831.
15. P.C. Jocelyn (1967) The standard redox potential of cysteine-cystine from the thiol-disulphide exchange reaction with glutathione and lipoic acid, *European Journal of Biochemistry*, **2,** 327–331.
16. F.B. Pruijn, G.R.M.M. Haenen and A. Bast (1991) Interplay between vitamin E, glutathione and dihydrolipoic acid in protection against lipid peroxidation, *Fat Science Technology*, **93,** 216–221.
17. Y.J. Suzuki, M. Tsuchlya and L. Packer (1991) Thioctic acid and dihydrolipoic acid are novel antioxidants which interact with reactive oxygen species, *Free Radical Research Communications*, **15,** 255–263.
18. B.A. Freeman and J.D. Crapo (1982) Biology of Diseases. Free radicals and tissue injury, *Laboratory Investigation*, **47,** 412–426.
19. D. Wang and D. Corbett (1990) Cerebral ischaemia, locomotor activity and spatial mapping, *Brain Research*, **533,** 78–82.
20. B.E. Mileson and R.D. Schwartz (1991) The use of locomotor activity as a behavioral screen for neuronal damage following transient forebrain ischemia in gerbils, *Neuroscience Letters*, **128,** 71–76.
21. W. Cao, J.M. Carney, A. Duchon, R.A. Floyd and M. Chevion (1988) Oxygen free radical involvement in ischemia and reperfusion injury to brain, *Neuroscience Letters*, **88,** 233–238.
22. J.W. Phillis (1994) A 'radical' view of cerebral ischemic injury, *Progress in Neurobiology*, **42,** 441–448.
23. X. Cao and J.W. Phillis (1994) α-Phenyl-tert-butyl-nitrone reduces cortical infarct and edema in rats subjected to focal ischemia, *Brain Research*, **644,** 267–272.
24. J.H.M. Prehn, C. Karkoutly, J. Nuglisch, B. Peruche and N.J. Krieglstein (1992) Dihydrolipoate reduces neuronal injury after cerebral ischemia, *Journal of Cerebral Blood Flow and Metabolism*, **12,** 78–87.
25. J.T. Greenamyre, M. Garcia-Osuna and J.G. Greene (1994) The endogenous cofactors, thioctic acid and dihydrolipoic acid, are neuroprotective against NMDA and malonic acid lesions of striatum, *Neuroscience Letters*, **171,** 17–20.
26. S. Stoll, H. Hartmann, S.A. Cohen and W.E. Müller (1993) The potent free radical scavenger α-lipoic acid improves memory in aged mice: putative relationship to NMDA receptor deficits, *Pharmacology Biochemistry and Behavior*, **46,** 799–805.
27. J. Peinado, H. Sies and T.P. Akerboom (1989) Hepatic lipoate uptake, *Archives of Biochemistry and Biophysics*, **273,** 389–395.
28. B.C. Scott, O.I. Aruoma, P.J. Evans, C. O'Neill, A. Vadervliet, C.E. Cross, H. Tritschler, and B. Halliwell (1994) Lipoic and dihydrolipoic acids as antioxidants. A critical evaluation., *Free Radical Research*, **20,** 119–133.
29. V.E. Kagan, A. Shvedova, E. Serbinova, S. Khan, C. Swanson, R. Powell and L. Packer (1992) Dihydrolipoic acid – a universal antioxidant both in the membrane and aqueous phase. Reduction of peroxyl, ascorbyl and chromanoxyl radicals, *Biochemical Pharmacology*, **44,** 1637–1649.

Accepted by Professor B. Halliwell