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Neuroprotection by α-Lipoic Acid in Streptozotocin-Induced Diabetes

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Abstract—Glial cells provide structural and metabolic support for neurons, and these cells become reactive to any insult to the central nervous system. The streptozotocin (STZ) rat model was used to study glial reactivity and the prevention of gliosis by α-lipoic acid (α-LA) administration. The expression of glial fibrillary acidic protein (GFAP), S100B protein, and neuron specific enolase (NSE) was determined as well as lipid peroxidation (LPO) and glutathione (GSH) levels in some brain tissues. Western blot analyses showed GFAP, S100B, and NSE levels significantly increased under STZ-induced diabetes in brain, and LPO level increased as well. Administration of α-LA reduced the expression both of glial and neuronal markers. In addition, α-LA significantly prevented the increase in LPO levels found in diabetic rats. GSH levels were increased by the administration of α-LA. This study suggests that α-LA prevents neural injury by inhibiting oxidative stress and suppressing reactive gliosis.

Key words: α-lipoic acid, glial fibrillary acidic protein, S100B protein, neuron specific enolase, diabetes

Diabetes mellitus is a common metabolic disorder, which affects the peripheral as well as the central nervous system [1]. Animal models of diabetes can contribute significantly into the effects of diabetes on the brain. Oxidative stress plays a central role in the pathogenesis and progression of diabetes and its complications. Enhanced formation of oxygen free radicals occurs in tissues during hyperglycemia [2]. These oxidant radicals contribute to increased neuronal death through protein oxidation, DNA damage, and peroxidation of membrane lipids [3, 4]. Both chemical and mechanical insults to the brain stimulate astrocyte proliferation with hypertrophy and the formation of glial filaments [5]. This phenomenon is called reactive gliosis. A key indicator of glial reactivity is the increased synthesis of glial fibrillary acidic protein (GFAP) and S100B protein [6]. GFAP is an intracellular intermediate filament protein of glial cells. It has been suggested that GFAP is essential for the formation of stable astrocytic processes in response to neuronal damage, and this may be critical for morphogenesis of the central nervous system [7]. Increases in GFAP are commonly used to examine the distributions of glial cells in response to neural injury [8]. Assay of S100B protein and neuron specific enolase (NSE) can provide qualitative information about the extent of brain injury and are sensitive markers of brain damage after stroke and cerebral hypoxia [9]. S100B protein and NSE are localized mainly in the central nervous system. NSE is a 78-kD glycolytic enzyme that is a soluble cytoplasmic protein localized principally in neurons. It was recently shown that NSE is a marker of neuronal damage in cerebral ischemia [10]. S100B is an acidic Ca^{2+}-binding protein present mainly in astrocytes that exerts paracrine trophic effects on several neuronal populations [11]. Blood S100B level is a good indicator for the assessment of patients with cerebral ischemia due to stroke [12]. We

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recently found that streptozotocin (STZ)-induced diabetes causes overexpression of glial markers (GFAP and S100B) both in brain and retinal tissues [13, 14].

Antioxidants are potential candidates for prevention or treatment of disorders involving oxidative damage. α-Lipoic acid (α-LA) has been described as a biological antioxidant and a potent free radical scavenger [15, 16]. α-Lipoate is taken up by the central nervous system and peripheral nerves. Numerous studies have demonstrated that α-LA is a potent antioxidant that can scavenge hydroxyl radical, hypochlorous acid, hydrogen peroxide, singlet oxygen, nitric oxide, and peroxynitrite [17, 18]. Recent studies indicate that α-lipoic acid has therapeutic potential in numerous neurodegenerative disorders, possibly as an antioxidant [17-19].

The present study was designed to extend the current information on antioxidant effects of α-lipoic acid as well as to determine its effects on neuronal and glial markers in rats after 45 days of uncontrolled diabetes.

MATERIALS AND METHODS

Forty-five adult male Wistar rats (weighing 200-250 g) obtained from the animal research unit of Firat University (Elazig) were used in this study. The rats were first divided into two groups. One group was made diabetic by a single intraperitoneal injection of STZ (Sigma, USA). STZ was dissolved in sodium citrate buffer (pH 4.5) and injected at a dose of 50 mg/kg body weight. Blood glucose levels were determined 3 days after STZ injection. Rats with a blood glucose concentration above 250 mg/dl were declared diabetic. Diabetic rats then were randomly assigned to two groups: one group received intraperitoneally (i.p.) 100 mg/kg α-LA once daily (α-LA group, $n = 15$) over a period of six weeks, while the other group received the vehicle alone (STZ group; $n = 15$). Age-matched control rats ($n = 15$) were injected with the citrate buffer vehicle used to dissolve STZ.

The rats were housed in a temperature-controlled room (22-25°C) with a 12/12 h light/dark cycle. Water and food were given *ad libitum*. The animals' body weight and the diabetic state were re-assessed after six weeks, just before sacrificing the animals. All protocols described were reviewed and approved by the local institutional committee for the ethical use of animals. Six-weeks STZ-diabetic rats and age-matched controls were killed by decapitation. The brain tissues were removed and the hippocampus, cortex, and cerebellum were dissected. Samples were used fresh or kept at −70°C.

Immunoblotting. Tissue samples were homogenized in 10 mM Tris-HCl (pH 7.4), 0.1 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 2 mM β-mercaptoethanol, 1% Triton X-100 containing protease inhibitor cocktail (Sigma) and centrifuged at 40,000*g* for 60 min at 4°C. Supernatants were collected, aliquoted, and stored at −70°C until use. Sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel electrophoresis was performed as described previously [20]. Samples and standard protein markers were separated on SDS-polyacrylamide gradient gel and proteins were transferred to nitrocellulose filters (Schleicher and Schuell, Inc., USA). Nonspecific binding sites were blocked by 1% bovine serum albumin. The blots were then incubated with primary antibodies (Santa Cruz Biotechnology, Inc., USA) at 1 : 2000 (anti-GFAP), 1 : 1000 (anti-S100B), and 1 : 1000 (anti-NSE) dilutions. After 1-h incubation, the blot was washed extensively in TBS-Tween (25 mM Tris-HCl, 0.2 mM NaCl, 0.1% Tween-20). Then membranes were incubated for 60 min at room temperature with peroxidase-conjugated goat-anti rabbit antibody (Santa Cruz Biotechnology, Inc.). The intensity of blots was measured in arbitrary units with commercial software after blot scanning (LabWorks 4.0, UVP, Inc., UK). Protein content was determined according to the Lowry procedure with a protein assay kit (Sigma). Tissue lipid peroxidation (as malondialdehyde + 4-hydroxyalkenals) was determined with LPO-586 kit (Oxis, Int. Inc., USA), the method being based on a reaction of N-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals at 45°C. Glutathione (GSH) levels were determined according to the method of Ellman [21].

Statistical analysis. The data are expressed as means ± SD; significance of differences between groups was evaluated by means of two-way analysis of variance in conjunction with Bonferroni's *t*-statistics, and p values <0.05 were considered statistically significant.

RESULTS

Streptozotocin injection induced a high degree of hyperglycemia compared to normal control animals (Table 1). Administration of α-LA significantly decreased the serum glucose levels compared to STZ-treated animals (Table 1). Lipid peroxidation (LPO) level significantly increased in brain tissues of STZ-treated rats compared with controls, while GSH level decreased significantly (Table 2). Brain tissue content of LPO was significantly decreased by the repeated administration of ALA in comparison to that in the STZ group (Table 2). In contrast, GSH level in hippocampus and cortex was increased by the administration of α-LA (Table 2).

GFAP content was significantly elevated in all brain tissues of STZ-induced diabetic rats compared to control. The degradation products of GFAP protein also increased in the all brain tissues of diabetic rats (figure, panel (a)). Similarly, the level of S100B protein, another glial marker, was significantly elevated by the STZ treatment compared to non-diabetic rats (figure, panel (b)). Since both GFAP and S100B protein are key indicators of glial reactivity, STZ-induced diabetes seems to induce

Table 1. Blood glucose, triglyceride, and total cholesterol levels (mean ± SD values are given for three groups of rats at the onset and at the end of the experiment)

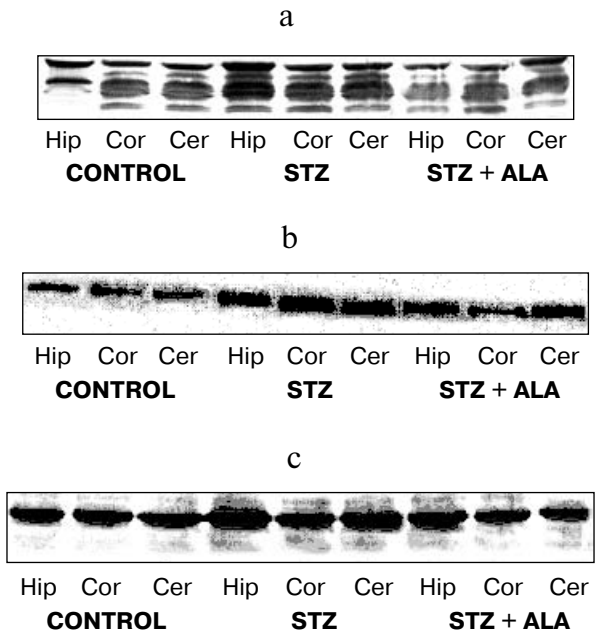
	Onset of study			End of study		
treatment	glucose, mg/dl	triglycerides, mg/dl	total cholesterol, mg/dl	glucose, mg/dl	triglycerides, mg/dl	total cholesterol, mg/dl
Control	120 ± 13	210 ± 22	102 ± 11	125 ± 12	212 ± 21	112 ± 11
STZ	125 ± 12	200 ± 22	110 ± 10	440 ± 50***	350 ± 37**	140 ± 14*
STZ + α-LA	122 ± 13	205 ± 21	112 ± 11	370 ± 52****	280 ± 29****	114 ± 12****

* $p < 0.05$ versus control.
** $p < 0.01$ versus control.
*** $p < 0.001$ versus control.
**** $p < 0.05$ versus STZ group.

Table 2. Results of densitometric analysis of glial fibrillary acidic protein (GFAP), S100B potein, and neuron specific enolase (NSE) expression (in arbitrary units) and the levels of lipid peroxidation (LPO) products (as malondialdehyde and 4-hydroxyalkenals, nmol/mg protein) and of reduced glutathione (GSH, μg/g protein) in brain tissues (mean ± SD)

Protein	Control	STZ	STZ + α-LA
Hippocampus			
GFAP	1.00 ± 0.00	1.75 ± 0.19***	1.05 ± 0.10[b]
S100B	1.00 ± 0.00	1.63 ± 0.15**	1.20 ± 0.10[a]
NSE	1.00 ± 0.00	1.96 ± 0.18***	1.05 ± 0.10[c]
LPO products	2.90 ± 0.28	5.10 ± 0.49***	3.60 ± 0.32[b]
GSH	397 ± 38	323 ± 31*	380 ± 39[a]
Cortex			
GFAP	1.00 ± 0.00	1.50 ± 0.16**	0.93 ± 0.08[b]
S100B	1.00 ± 0.00	1.90 ± 0.18***	0.88 ± 0.08[c]
NSE	1.00 ± 0.00	1.78 ± 0.18***	1.19 ± 0.10[b]
LPO products	3.40 ± 0.38	4.80 ± 0.50**	3.50 ± 0.38[b]
GSH	400 ± 42	337 ± 34*	405 ± 42[a]
Cerebellum			
GFAP	1.00 ± 0.00	1.67 ± 0.17***	1.10 ± 0.10[b]
S100B	1.00 ± 0.00	1.78 ± 0.17***	1.26 ± 0.13[a]
NSE	1.00 ± 0.00	1.85 ± 0.19***	1.30 ± 0.14[b]
LPO products	3.18 ± 0.38	4.20 ± 0.48*	3.00 ± 0.24[b]
GSH	410 ± 38	330 ± 35*	370 ± 47

* $p < 0.05$ versus control.
** $p < 0.01$ versus control.
*** $p < 0.001$ versus control.
[a] $p < 0.05$ versus STZ groups.
[b] $p < 0.01$ versus STZ groups.
[c] $p < 0.001$ versus STZ groups.



Western blot analysis of glial fibrillary acidic protein (a), S100B protein (b), and neuron specific enolase (c) from hippocampal (Hip), cortical (Cor), and cerebellar (Cer) homogenates of control, STZ, and (STZ + α-LA) groups

glial hyperactivity. Furthermore, STZ-induced diabetes led to increase in the levels of NSE (figure, panel (c)). Daily administration of α-LA significantly prevented the increase in glial and neural markers in diabetic rats (Table 2). There was a significant correlation between GFAP and LPO levels in hippocampus and cortex of diabetic rats ($r = 0.675$, $p < 0.01$; $r = 0.603$, $p < 0.05$, respectively).

DISCUSSION

Neuronal degeneration might be related to production of free radicals due to the impairment of mitochondrial function and antioxidant status in diabetes [22]. It has been reported that oxidative stress increases neuronal death that, in turn, contributes to the neuropathology associated with diabetes [23]. In agreement with the previous studies, herein we found that LPO level was elevated in brain tissues of STZ-treated rats while the amount of GSH decreased [24, 25]. GSH is an antioxidant found in all animal cells. It reacts with the free radicals and can protect cells from singlet oxygen, hydroxyl radical, and super oxide radical damage [26]. In agreement with the previous studies, here we found that α-LA reduced the glucose levels in diabetic rats [27]. This process may proceed through a mechanism by which α-LA enhances insulin-stimulated glucose metabolism in skeletal muscle [27]. The present findings show that hyperglycemia induces reactive gliosis with the involvement of a free radical-mediated pathway that activates glial cells. The positive correlation between glial markers and LPO level in hippocampus and cortex of diabetic rats, observed in our study, supports the above hypothesis. Astrocytes in the central nervous system on injury induced by trauma, disease, genetic disorders, or chemical insult become reactive and respond in a typical manner termed astrogliosis [28]. Astrogliosis is also characterized by rapid synthesis of S100B protein and GFAP intermediate filaments [13]. It may be reasoned that astrocytic response might be beneficial for neuronal survival. However, it is known that activated glial cells are able to release cytokines, complement components, and free radicals. In addition, elevated S100B level is involved in the pathogenesis of neurodegenerative processes, possibly through oxidative stress mechanisms [29].

In the present study, we found that administration of α-LA reduces the LPO levels in brain regions of diabetic rats (Table 2). This clearly indicates that α-LA prevents diabetes-induced lipid damage in the brain tissue of rats and suggests that free radical scavenging and anti-oxidative properties of α-LA could explain, at least partially, the α-LA afforded protection against hyperglycemia-induced neural damage. This is consistent with the previous studies where the protective effect of α-LA against toxic neuronal injury was attributed to its free radical scavenging properties [15, 17, 30]. Administration of α-LA significantly increased GSH level in brain tissue (Table 2). This suggests that the antioxidant property of α-LA was responsible for protection against the oxidative stress, in part by increasing the endogenous defensive capacity of the brain to oxidative stress induced by STZ [31]. α-LA may be effective both in prevention and treatment of oxidative stress in a number of models or clinical conditions [32]. Antioxidants such as α-LA, glutathione, and vitamins (A, E, C) detoxify hydrogen peroxide, hydroxyl radicals, peroxyl radicals, and reactive nitrogen species [15, 16, 33]. Furthermore, they increase the activity of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase. High oxidative stress in diabetes results in damage to macromolecules such as nucleic acids, proteins, and lipids [2-4]. These oxidized macromolecules cause cell dysfunction and necrosis, leading to functional and structural degeneration of the central nervous system. Thus, antioxidants can be potentially beneficial in prevention of cardiovascular and cerebrovascular complications of diabetes by reducing the accumulation of reactive oxygen species (ROS). The beneficial effects of α-LA treatment were also manifested by the down regulation of glial reactivity. To the best of our knowledge, the present findings are the first that show the reduction both of glial and neuronal markers in different brain tissues of diabetic rats by α-LA administration. Since increased levels of glial and neuronal markers are associated with the severity of cerebral damage, the down

regulation of these markers is considered to favor the restoration of the affected neuronal circuits. Gonzalez-Parez et al. [34] found that treatment with alpha lipoic acid plus vitamin E mixture in a thromboembolic stroke model in rats significantly reduced reactive gliosis and this effect was attributed to their antioxidant ability. It is likely that several mechanisms account for the astrocytic reaction in diabetes, one such factor being a free radical insult to the central nervous system [35]. The present findings show that the increase in glial markers during hyperglycemia and their down regulation by α-LA antioxidant is a direct response of astrocytes to oxidative stress. Furthermore, decreased NSE level may indicate that neuronal injury induced by hyperglycemia is prevented by the administration of α-LA. In addition to its antioxidant properties, α-LA can be neuroprotective *in vivo* through suppression of glial reactivity. Since chronic reactive gliosis exacerbates diabetic neuropathy, the administration of α-LA can prevent neuropathy by reducing both oxidative stress and glial hyperactivity.

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REFERENCES

1. Biessels, G. J., Kappelle, A. C., Bravenboer, B., Erkelens, D. W., and Gispen, W. H. (1994) *Diabetologia*, **37**, 643-650.
2. Baydas, G., Canatan, H., and Turkoglu, A. (2002) *J. Pineal Res.*, **32**, 225-230.
3. Hawkins, C. L., and Davies, M. J. (2001) *Biochim. Biophys. Acta*, **1504**, 196-219.
4. Luxford, C., Dean, R. T., and Davies, M. J. (2000) *Chem. Res. Toxicol.*, **13**, 665-672.
5. Klunker, P. E., and Kraig, R. P. (1997) *J. Cerebr. Blood Flow Metab.*, **17**, 26-43.
6. Baydas, G., Reiter, R. J., Yasar, A., Tuzcu, M., Akdemir, I., and Nedzvetskii, V. S. (2003) *Free Rad. Biol. Med.*, **35**, 797-804.
7. Liedtke, W., Edelmann, W., Bieri, P. L., Chiu, F. C., Cowan, N. J., Kucherlapati, R., and Raine, C. S. (1996) *Neuron*, **17**, 607-615.
8. Baydas, G., Reiter, R. J., Nedzvetskii, V. S., Yasar, A., Tuzcu, M., Ozveren, F., and Canatan, H. (2003) *Toxicol. Lett.*, **137**, 169-174.
9. Persson, L., Hardemark, H. G., Gustafsson, J., Rundstrom, G., Mendel-Hartvig, I., Esscher, T., and Pahlman, S. (1987) *Stroke*, **18**, 911-918.
10. Hatfield, R. H., and McKernan, R. M. (1992) *Brain Res.*, **577**, 249-252.
11. Kligman, D., and Marshak, D. R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7136-7139.
12. Lynch, J. R., Blessing, R., White, W. D., Grocott, H. P., Newman, M. F., and Laskowitz, D. T. (2004) *Stroke*, **35**, 57-63.
13. Baydas, G., Nedzvetskii, V. S., Tuzcu, M., Yasar, A., and Kirichenko, S. V. (2003) *Eur. J. Pharmacol.*, **462**, 67-71.
14. Baydas, G., Tuzcu, M., Yasar, A., and Baydas, B. (2004) *Acta Diabetol.*, in press.
15. Packer, L., Tritschler, H. J., and Wessel, K. (1997) *Free Rad. Biol. Med.*, **22**, 359-378.
16. Zhang, L., Xing, G. Q., Barker, J. L., Chang, Y., Maric, D., Ma, W., Li, B. S., and Rubinow, D. R. (2001) *Neurosci. Lett.*, **312**, 125-128.
17. Packer, L., Kraemer, K., and Rimbach, G. (2001) *Nutrition*, **17**, 888-895.
18. Haenen, G. R., and Bast, A. (1991) *Biochem. Pharmacol.*, **42**, 2244-2246.
19. Packer, L., Witt, E. H., and Tritschler, H. J. (1995) *Free Rad. Biol. Med.*, **19**, 227-250.
20. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
21. Ellman, G. L. (1959) *Arch. Biochem. Biophys.*, **82**, 70-77.
22. Yorek, M. A. (2003) *Free Rad. Res.*, **37**, 471-480.
23. Greene, D. A., Stevens, M. J., Obrosova, I., and Feldman, E. L. (1999) *Eur. J. Pharmacol.*, **375**, 217-223.
24. Raza, H., Ahmed, I., John, A., and Sharma, A. K. (2000) *J. Biochem. Mol. Toxicol.*, **14**, 131-139.
25. Celik, S., Baydas, G., and Yilmaz, O. (2002) *Cell. Biochem. Funct.*, **20**, 67-71.
26. Sharma, M., Rai, K., Sharma, S. S., and Gupta, Y. K. (2000) *Pharmacology*, **60**, 90-96.
27. Jacob, S., Streeper, R. S., Fogt, D. L., Hokama, J. Y., Tritschler, H. J., Dietze, G. J., and Henriksen, E. J. (1996) *Diabetes*, **45**, 1024-1029.
28. Zhu, W., Umegaki, H., Shinkai, T., Kurotani, S., Suzuki, Y., Endo, H., and Iguchi, A. (2003) *J. Gerontol. A. Biol. Sci. Med. Sci.*, **58**, 117-122.
29. Corvino, V., Businaro, R., Geloso, M. C., Bigini, P., Cavallo, V., Pompili, E., Mennini, T., Fumagalli, L., and Michetti, F. (2003) *Neurochem. Res.*, **28**, 341-345.
30. Baydas, G., Yilmaz, O., Celik, S., Yasar, A., and Gursu, M. F. (2002) *Arch. Med. Res.*, **33**, 515-519.
31. Sharma, M., and Gupta, Y. K. (2003) *Eur. Neuropsychopharmacol.*, **13**, 241-247.
32. Roy, S., Sen, C. K., Tritschler, H. J., and Packer, L. (1997) *Biochem. Pharmacol.*, **53**, 393-399.
33. Zaidi, S. M., and Banu, N. **(**2004) *Clin. Chim. Acta*, **340**, 229-233.
34. Gonzalez-Perez, O., Gonzalez-Castaneda, R. E., Huerta, M., Luquin, S., Gomez-Pinedo, U., Sanchez-Almaraz, E., Navarro-Ruiz, A., and Garcia-Estrada, J. (2002) *Neurosci. Lett.*, **321**, 100-104.
35. Kaneko, K., Nakamura, A., Yoshida, K., Kametani, F., Higuchi, K., and Ikeda, S. (2002) *Free Rad. Res.*, **36**, 303-306.