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Alpha-lipoic acid does not alter stress protein response to acute exercise in diabetic brain

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Heat shock proteins (HSPs) are molecular chaperones which may act protective in cerebrovascular insults and peripheral diabetic neuropathy. We hypothesized that alpha-lipoic acid (LA), a natural thiol antioxidant, may enhance brain HSP response in diabetes. Rats with or without streptozotocin-induced diabetes were treated with LA or saline for 8 weeks. Half of the rats were subjected to exhaustive exercise to investigate HSP induction, and the brain tissue was analyzed. Diabetes increased constitutive HSC70 mRNA, and decreased HSP90 and glucose-regulated protein 75 (GRP75) mRNA without affecting protein levels. Exercise increased HSP90 protein and mRNA, and also GRP75 and heme oxygenase-1 (HO-1) mRNA only in non-diabetic animals. LA had no significant effect on brain HSPs, although LA increased HSC70 and HO-1 mRNA in diabetic animals and decreased HSC70 mRNA in non-diabetic animals. Eukaryotic translation elongation factor-2, essential for protein synthesis, was decreased by diabetes and suggesting a mechanism for the impaired HSP response related to translocation of the nascent chain during protein synthesis. LA supplementation does not offset the adverse effects of diabetes on brain HSP mRNA expression. Diabetes may impair HSP translation through elongation factors related to nascent chain translocation and subsequent responses to acute stress. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS — antioxidant; brain; diabetes; exercise; lipoic acid

INTRODUCTION

Heat shock proteins (HSPs) perform pivotal roles under stressful conditions such as temperature changes and oxidative stress by protecting against tissue injury through maintenance of synthesis and proper conformation and repair of proteins, and by promoting healing of injured tissue.[1] Furthermore, HSPs are thought to play a protective role in many brain-related pathological conditions such as ischemia and reperfusion and diabetes and its complications.[1–3]

Some HSPs, such as the 70 kDa HSP72, are strongly inducible by elevated temperatures and protect brain synaptic structures during heat shock and brain ischemia.[4,5] In diabetes, the expression of HSPs can be impaired.[1,6] However, experimental evidence suggests a protective effect of HSP72 in peripheral diabetic neuropathy,[7] although decreased[6,8] or unchanged[9] levels have also been described in diabetic tissue, including the brain.[10] These discrepancies are likely to be tissue-dependent and related to the duration or severity of diabetes, or other yet unidentified factors.

Exhaustive physical exercise can induce oxidative damage in tissues[11] including the brain,[12] which may have an unfavorable effect in the diabetic brain due to alterations in the redox balance[13] and increased oxidative stress,[14] Oxidative stress,[1,15,16] and acute and chronic exercise also induce HSP synthesis in various tissues,[6,17] including the brain.[18] The type[19,20] and intensity of exercise[21,22] also influence HSP responses.

Alpha-lipoic acid (LA) is a potent thiol antioxidant that acts as a cofactor in the pyruvate dehydrogenase complex and has shown to be beneficial in conditions associated with increased oxidative stress, such as peripheral diabetic neuropathy.[23] Moreover, LA increased HSPs in the diabetic kidney and in the skeletal muscle of non-diabetic rats.[24,25] As such, LA may be useful for preventing exercise-induced tissue damage. We recently reported that a 5-week oral LA

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supplementation increased the resting levels of HSP90 and post-exercise recovery levels of HSP72 in the skeletal muscle in horse.[26] However, the effects of LA on brain HSP response in diabetes are not known.

In a recent study, we used endurance training as a tool to enhance brain HSP response and showed that exercise training induced HSP response in non-diabetic animals, but not in diabetic animals at the protein level.[10] As a continuation of our investigations, the present study was set forth to test the potency of LA for enhancing brain HSP responses in diabetes, because increased levels of HSPs may act beneficial for brain by increasing resistance to stress-induced neuronal damage.[5] To induce acute physiological metabolic stress and HSP induction, a bout of exhaustive exercise was used with or without LA pre-treatment to evaluate if LA would exert a favorable effect on brain HSP response under a sudden oxidative insult. Furthermore, to gain insight on the mechanisms of brain protein synthesis potentially disrupted in diabetes, we measured the levels of eukaryotic elongation factors 1a and 2 (eEF-1a and eEF-2), which are key regulators of mRNA translation.

MATERIALS AND METHODS

Animals

Twelve weeks old male Wistar rats ($n = 48$) were maintained at $22 \pm 2^{\circ}$C with 12:12 h light–dark cycles and access to standard chow and water *ad libitum*. Animal care and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985). The experimental protocol (No. 17/98) was approved by the Ethics Committee for laboratory animal research of University of Kuopio.

Preparation of diabetic rats

The animals were randomly assigned to non-diabetic control ($n = 24$) and diabetic ($n = 24$) groups. As an experimental model of type 1 diabetes, a single intraperitoneal injection of streptozotozin (60 mg kg^{-1}, prepared in 0.1 M citrate buffer, pH 4.5) was administered to destroy animals' pancreatic beta cells.[6] Glucosuria was confirmed using test strips (BM-Test-5L, Boehringer-Mannheim, Germany) 1 week after the injection, and repeated weekly through the study. In addition, blood glucose levels were measured at the end of the study in truncal blood collected immediately after decapitation by a commercial kit (Gluco-quant Glucose/HK, Boehringer Mannheim) using the hexokinase reaction. Animals with sustained diabetes (glucosuria of at least 20 mmol L^{-1} two weeks after the injection) and the non-diabetic control animals were further divided into groups with ($n = 12$) or without ($n = 12$) LA supplementation.

LA was administered orally (150 mg kg^{-1} day^{-1}) for 8 weeks, while the control animals received a matched volume of phosphate buffered saline. Half of the LA-supplemented and non-supplemented rats were sacrificed at rest by decapitation and the other half immediately after acute exhaustive exercise ($n = 6$ per group). Treadmill exercise to exhaustion was performed as described.[27] A mild electrical shock deterrent was used intermittently when necessary to coerce the rats to run. Exhaustion was identified as the loss of righting reflex of the animals when laid on their back. Whole brain (cerebrum) of the animals were quickly removed, rinsed in ice-cold saline, blotted, placed in liquid nitrogen, and stored at -75°C until use.

Analysis of stress proteins and eukaryotic elongation factors by Western blot

Standard Western blot techniques were used to analyze protein expression in brain, as previously described.[10] First, the frozen brains were pulverized under liquid nitrogen with a mortar and sonicated in a buffer containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 nM EDTA, 20 mM HEPES, 5 μM DTT, and 5 μM PMSF at 4°C. Protein levels of the brain extracts were quantified by bicinchoninic acid (BCA) assay (Pierce, Rockford, Illinois, USA). Equal amounts of protein (30 μg) were electrophoresed together with molecular weight markers on SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Whatman, Kent; UK). Next, after blocking with 5% fat-free milk solution at 37°C for 60 min, the membranes were treated with the following antibodies (all from StressGen, British Columbia, Victoria, Canada) recognizing the 60 kDa HSP (HSP60), HSP72 and the constitutive cognate form of the 70 kDa HSP (HSC70), the 90 kDa HSP (HSP90) and the glucose-regulated protein 75 (GRP75). Polyclonal Abs for eEF-1a and eEF-2 were purchased from Santa-Cruz Biotechnology (Santa Cruz, California, USA).

Horseradish peroxidase-conjugated anti-mouse (Santa Cruz) and anti-rat immunoglobulins (Zymed Laboratories, San Francisco, California, USA) were used as secondary Abs, when appropriate. A mouse monoclonal Ab for beta-Actin (Sigma, St. Louis, Missouri, USA) served as an endogenous loading control. The membranes were developed using an infrared imaging system (Odyssey, LI-COR Bioscience, Lincoln, Nebraska, USA) and quantified using image-analysis software (ScionCorp, Frederick, Maryland, USA). For clarity, the data were normalized to beta-Actin and expressed relative to values from the untrained non-diabetic, non-LA-supplemented control group.

Analysis of gene expression

A quantitative real-time RT-PCR was applied to analyze mRNA levels of HSP60, HSC70, HSP72, HSP90, GRP75 in brain as described.[10] Briefly, 100 mg of tissue was first homogenized with Ultra-Turrax (Janke and Kunkel, Germany) and total cellular RNA was isolated using Eurozol reagent according to manufacturer's instructions (Euroclone, West York, UK). Nucleic acid concentrations were determined by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and their

integrity was checked with gel electrophoresis. One microgram of RNA per sample was then converted to cDNA using SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, California, USA) and oligo(dT) primers (Promega, Madison, Wisconsin, USA).

For PCR primer design, the annotated nucleotide sequences were retrieved from the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA), and BLAST searches were performed to identify unique stretches of nucleotide sequence and not to amplify genomic DNA. The primers were as follows and are shown in 5′–3′ orientation: HSP60 forward primer (-F) AAAGCTGAACGAGCGACTTG and reverse primer (-R) ATCACTTGTCCCTCCAACCTTC; HSC70-F AGCACCCAGGCCAGTATTG and HSC70-R CAGCATTCAACTCCTCAAATCG; HSP72-F CAACTGGCTTGACCGAAACC and HSP72-RAGCGCAAGCCTAGTCCAC TTC; HSP90-F GTACGAAA CAGCACTCCTGTCTTC and HSP90-R ATCCTCATCAA TACCTAGACCAAGC; GRP 75-F ACGAGGATGCCCAAGGTTC and GRP75-R TGAAT GGCAGCTCCAATGG; HO-1-F GGAAGGCTTTAAGCTG GTGATG and HO-1-R GGTTCTGCTTGTTTCGCTCTATC; CypB-F GCCTTAGCTACAGGAGAGAAAGGA and CypB-R TCCACCCTGGATCATGAAGTC.

For PCR analysis, the samples were amplified on Mx3000P System (Stratagene, La Jolla, California, USA) using Brilliant SYBR Green Master Mix (Stratagene) and 200 nM of gene-specific primers with a 10 min pre-incubation at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 60°C, and 25 s at 72°C. The data were normalized to cyclophilin B (CypB) mRNA and processed as relative units. Unique amplification and absence of primer-dimers were evaluated by melt curve analysis.

Statistics

Calculations were done using SPSS software (Chicago, IL, USA). Effect of diabetes and LA supplementation, and exercise and LA in diabetic and non-diabetic animals was tested with two-way ANOVA. Statistical significance was set at $p < 0.05$. Data are represented as mean ± SD unless stated otherwise.

RESULTS

Diabetes increased mRNA levels of the constitutively expressed HSC70 ($p = 0.042$) and decreased HSP90 ($p = 0.046$) and GRP75 ($p = 0.039$) mRNAs, but had no effect on the respective protein levels (Figures 1,3 and 4). In addition, diabetes decreased eEF-2 protein levels ($p = 0.001$), whereas those of eEF-1 were not affected by any of the experimental conditions (Figures 6 and 7).

To study HSP response after sudden metabolic stress, acute exercise was not found to affect HSP72 and HSP60 mRNA expression or protein levels (Figures 1 and 2), but to increase HSP90 protein levels ($p = 0.012$) and HSP90 mRNA ($p = 0.044$), and also GRP75 mRNA ($p = 0.042$) in non-diabetic animals (Figures 3 and 4). In addition, HO-1 mRNA expression was significantly increased by exercise in non-diabetic animals ($p = 0.005$), but not in diabetic animals (Figure 5).

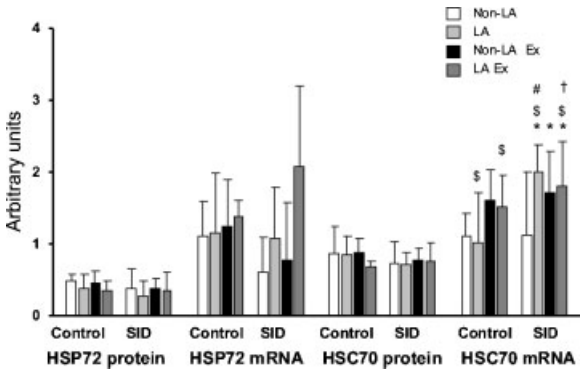

Figure 1. Effect of streptozotocin-induced diabetes (SID), 8 weeks of alpha-lipoic acid supplementation (LA) and exhaustive exercise (Ex) on HSP72 and HSC70 protein and mRNA levels in rat brain. Values are mean ± SD. The acronyms for the groups ($n = 6$ per group) are as follows: without LA supplementation (Non-LA), with LA supplementation (LA), exhaustive exercise without LA (Non-LA Ex), and exhaustive exercise with LA supplementation (LA Ex). For the difference due to SID: $^{*}p < 0.05$; due to LA: $^{\$}p < 0.05$; interaction between diabetes and LA: $^{\#}p < 0.05$; interaction between Ex and LA in diabetic animals: $^{\dagger}p < 0.05$. For the representative Western blots, see Figure 7.

LA supplementation increased HSC70 mRNA expression in diabetic animals ($p = 0.015$; interaction without exercise $p = 0.032$, and with exercise $p = 0.021$), but decreased in non-diabetic controls ($p = 0.012$) (Figure 1). On the other hand, LA supplementation had no effect on the levels of all analyzed proteins as they remained unchanged (Figures 1–7). Similarly, HSP90 and GRP75 mRNA and protein levels (Figures 3 and 4), and HO-1 mRNA expression were not affected by LA supplementation, except for the slightly increased HO-1 mRNA expression in diabetic animals ($p = 0.045$), suggesting a possible interaction of LA with diabetes (Figures 3–5, 7).

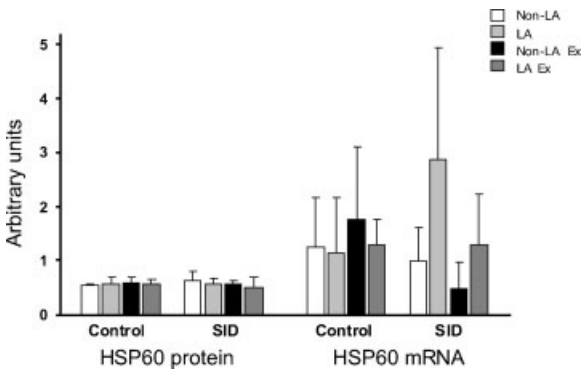

Figure 2. Effect of streptozotocin-induced diabetes (SID), 8 weeks of alpha-lipoic acid supplementation (LA) and exhaustive exercise (Ex) on HSP60 protein and mRNA levels in rat brain. Values are mean ± SD. Groups are as in Figure 1. For the representative Western blots, see Figure 7.

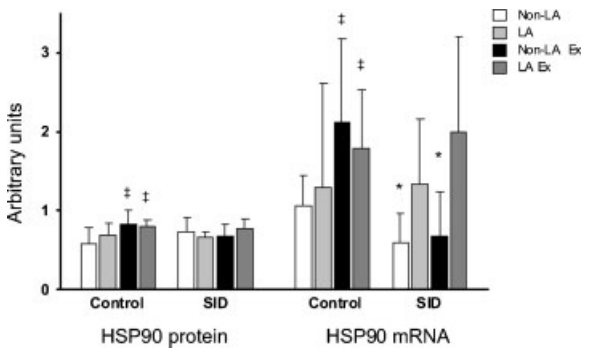

Figure 3. Effect of streptozotocin-induced diabetes, 8 weeks of alpha-lipoic acid supplementation and exhaustive exercise on HSP90 protein and mRNA levels in rat brain. Values are mean ± SD. Groups are as in Figure 1. For the difference due to SID: $^{*}p < 0.05$; due to exhaustive exercise: $^{\ddagger}p < 0.05$. For the representative Western blots, see Figure 7.

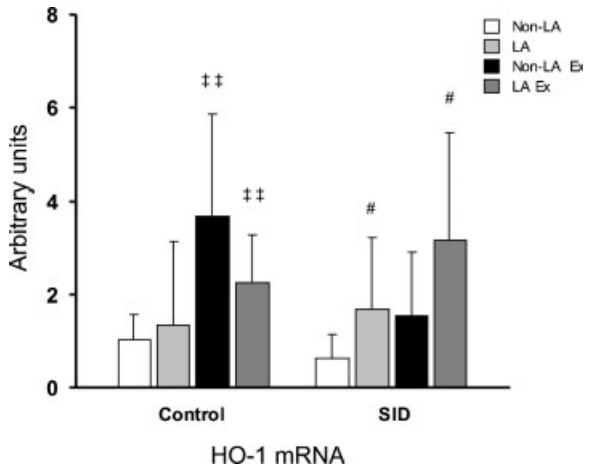

Figure 5. Effect of streptozotocin-induced diabetes, 8 weeks of alpha-lipoic acid supplementation and exhaustive exercise on HO-1 mRNA levels in rat brain. Values are mean ± SD. Groups are as in Figure 1. For the difference due to exhaustive exercise: $^{\ddagger\ddagger}p < 0.01$; for the difference due to LA: $^{\#}p < 0.05$.

DISCUSSION

Acute exercise induced brain HSP90 protein and mRNA synthesis, and also HO-1 and GRP75 mRNA expression in non-diabetic animals, but not in diabetic animals. Long-term LA supplementation increased HSC70 and HO-1 mRNA expression in diabetic animals, and decreased HSC70 mRNA in non-diabetic controls, but no concomitant effects were found at the protein level. LA supplementation also did not modify the HSP response to exhaustive exercise. To our knowledge, this is the first study reporting the influence of LA on brain HSP expression.

Despite the increased mRNA expression of some of the investigated HSPs, a somewhat weak overall HSP response to exercise was observed at the protein level, which may also reflect the time the animals were sacrificed after the exercise thus allowing insufficient time for protein synthesis. HSPs are primarily cytoprotective components and appear to be induced via highly regulated signaling cascades, including the major mitogen-activated protein kinases (MAPK) and protein kinase B (PKB/Akt), which can be impacted by exercise.[28] However, these pathways were not investigated in the present study. For some reason, the inducible HSP72 remained unchanged after exercise also at the mRNA level, which may indicate that this HSP does not elicit a strong response to acute metabolic stress in the brain, whereas upregulation of HSP90 at the mRNA and protein level implies a protective response in non-diabetic animals, but was impaired by diabetes. By itself, HSP90 is abundantly expressed in the cytosol and responsible for catalyzing the interaction with several substrate proteins and co-chaperones involved in cell regulation and intracellular signaling. Our findings on exercise-induced brain HSP synthesis in non-diabetic rats, but not in diabetic rats, are consistent with our previous study in which endurance exercise training induced brain HSPs in non-diabetic animals and this effect was blunted in diabetes.[10]

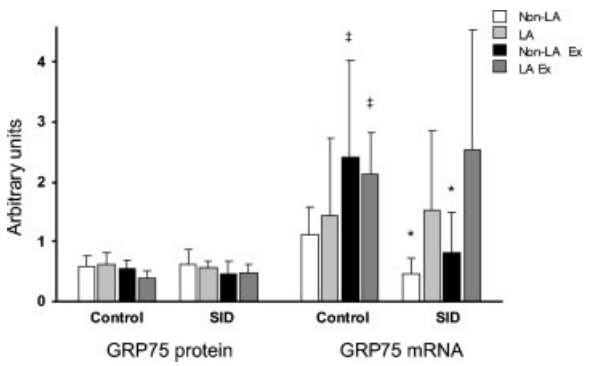

Figure 4. Effect of streptozotocin-induced diabetes, 8 weeks of alpha-lipoic acid supplementation and exhaustive exercise on GRP75 protein and mRNA levels in rat brain. Values are mean ± SD. Groups are as in Figure 1. For the difference due to SID: $^{*}p < 0.05$; due to exhaustive exercise: $^{\ddagger}p < 0.05$. For the representative Western blots, see Figure 7.

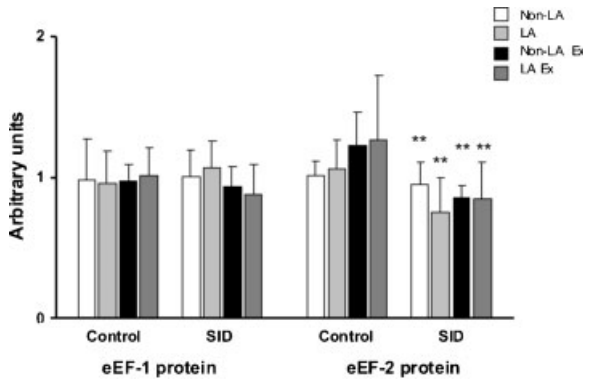

Figure 6. Effect of streptozotocin-induced diabetes, 8 weeks of alpha-lipoic acid supplementation and exhaustive exercise on eEF-1 and eEF-2 protein levels in rat brain. Values are mean ± SD. Groups are as in Figure 1. For the difference due to SID: $^{**}p < 0.01$. For the representative Western blots, see Figure 7.

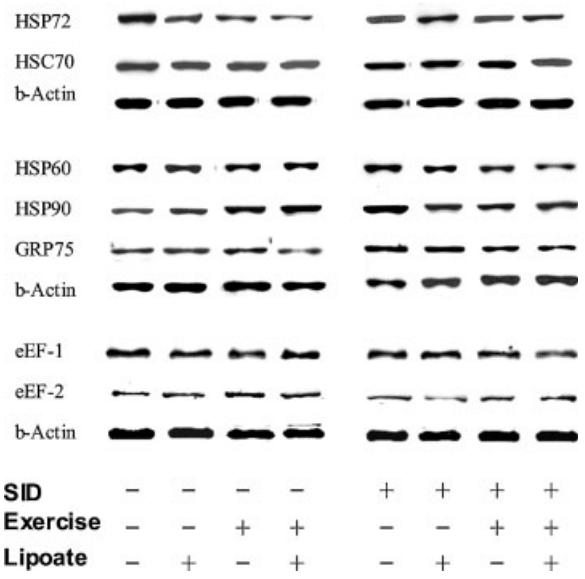

Figure 7. Representative Western blots for the expression of cytoprotective proteins. The study groups are as in Figure 1 indicated with +/− symbol when appropriate. Beta-actin was used as a loading control.

Despite its crucial role as a mediator of antioxidant and tissue-protecting actions, little is known about the functional significance of HO-1 in diabetes. The present study showed unaltered HO-1 mRNA expression in the diabetic brain, whereas decreased levels have previously been reported in the skeletal muscle of type 2 diabetic subjects and increased levels in the peripheral-blood lymphocytes.[3,29] and in diabetic rat kidney.[25] Overall, the information on HO-1 in diabetes remains vague.

The levels of HSC70, HSP72 and HSP60 protein or mRNA did not significantly change in response to exercise in the present study, which is in agreement with recent reports,[30–32] although some studies have reported increased HSC70 levels in brain after severe exercise.[33] Also in human subjects, the brain is capable of releasing HSP72 *in vivo* after prolonged exercise,[18] which may attenuate the overall HSP levels in the brain tissue.

Interestingly, LA supplementation increased HO-1 mRNA expression in diabetic animals. LA has previously been shown to increase HO-1 levels *in vitro*,[34] but reports in diabetic tissue are scarce. LA also decreased HSC70 mRNA in non-diabetic rats, but did not affect protein levels.

No effect of LA on the HSP response to acute exercise was found. Earlier, high LA doses have shown to enhance HSP formation via increased disulfide formation in certain target proteins at least in tissues other than brain.[24,35] However, our findings suggest that LA supplementation has only limited effects on brain HSPs in non-diabetic and diabetic animals. It is, therefore, likely that the effects of LA are tissue and dose specific. On the other hand, we found that LA did not compromise the HSP induction, which is in contrast to reports using other antioxidants.[36] For example, vitamin C and E have previously been shown to attenuate or completely inhibit the exercise-induced increase of HSPs in circulation and in the skeletal muscle.[37,38]

Some pathological conditions such as diabetes have been related with changes in the elongation factors,[39] which are transcription factors crucial for nascent peptide translocation in the ribosome. Consistently, eEF-2 is a key component of the translational machinery and potentially responsible for the incomplete mRNA translation into protein in diabetes. As such, diabetes may compromise stress protein response through impaired levels of eEF-2. Our findings also support this hypothesis as a defective mRNA translation in diabetes may contribute to the impaired HSP synthesis. Unfortunately, we could not analyze any functional modifications of this protein, such as phosphorylation. With respect to LA, this had no effect on the levels of elongation factors, which is in line with the present data on HSP induction. Similarly, we observed that exercise had no effect on eEF levels, also supported by a previous report.[40] Depressed levels of the key elongation factor in diabetes are suggestive for impaired HSP induction through decreased mRNA translation into the respective protein.

Physical exercise has numerous favorable effects on health. Among its therapeutic effects, exercise can increase HSP expression, perhaps contributing to the improved outcomes associated with exercise and diabetes. Therapy directed at raising HSPs may not only limit the development of diabetes, but also reduce diabetes-associated morbidity and mortality. Findings of the present study suggest that, except for increased HSC70 mRNA expression, oral LA treatment has no significant effect on brain HSP synthesis. On the other hand, LA does not seem to have negative effects via blunted HSP response, as has been reported with some antioxidant supplementations.[38] Furthermore, Ristow *et al*. have recently reported that antioxidant supplements may, in fact, prevent the induction of molecular regulators of insulin sensitivity and endogenous antioxidant defense by physical exercise in humans.[41] Further studies are needed to define the potential benefits of LA on brain cytoprotection as well as to determine the dose and timing for maximum effectiveness.

CONFLICT OF INTEREST

None known.

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