Alpha-Linolenic Acid Supplementation is Associated with Changes in Inflammatory Markers and Endoplasmic Reticulum Stress in Diabetic Rats

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Abstract

Background: The aim of this study was to evaluate the effects of α-linolenic acid (ALA) supplementation on inflammation and endoplasmic reticulum stress (ERS) in streptozotocin induced diabetic rats.

Methods: We studied 40 Wistar rats divided into four groups: control, control+ALA, diabetes and diabetes+ALA. The +ALA groups received supplementation of 3 g of ALA from flaxseed, daily for a period of 8 weeks. Measurements of blood glucose levels, serum insulin, lipid profile, serum cytokines (TNF-α, IL-6 and INF-γ) and body weight were performed before and after the ALA supplementation. Protein expression of AKT, IRE1-α, BIP, and CHOP were evaluated in liver tissue.

Results: The diabetes+ALA group had lower liver and greater epidydimal adipose tissue weight in relation to the diabetes group, besides no difference in total body weight. Diabetes+ALA group showed lower glucose and triglyceride levels after the ALA supplementation compared to diabetes group, but no difference in total cholesterol. Insulin levels were significantly lower in the diabetic group compared to the control, but there was no difference between the control group and diabetes+ALA group. The ALA supplementation did not determine significant changes in TNF-α and IL-6 levels. However, the diabetic+ALA group showed a decrease in the serum INF-γ levels after the supplementation period. We also observed increased expression of HSP-90 and HSP-70 in hepatic tissue of the diabetes+ALA animals compared to the diabetes group, associated to lower BIP and XBP-1 protein expression. We also observed a decrease in AKT protein expression in the diabetes+ALA group.

Conclusion: In conclusion, supplementation of ALA reduced blood glucose and serum triglyceride levels associated to a reduction in systemic inflammation and it was also able to influence important pathways involved in the modulation of ERS.

Keywords: Alpha-linolenic acid; Endoplasmic reticulum stress; Inflammation; Diabetes mellitus; Streptozotocin; Rats

Abbreviations: ALA: Alpha Linolenic Acid; ERS: Endoplasmic Reticulum Stress; IRE1-α: Inositol Requiring Enzyme 1-α; CHOP: C/EBP Homologous Protein; XBP-1: X-box Binding Protein 1; BIP: Glucose Regulating Protein 78; HSP: Heat Shock Protein; IL-6: Interleukine-6; AKT: Protein Quinase B; TNF-α: Tumor Necrosis Factor-α; INF-γ: Interferon-γ

Introduction

A state of chronic and subclinical inflammation often accompanies excessive lipid accumulation in adipose and liver tissue, as evidenced by changes in inflammatory cells and biochemical markers of inflammation [1,2]. The endoplasmic reticulum (ER) is well known as the cellular organelle responsible for protein maturation and growing and evidence suggests that the ER also acts as a principal stress sensor [3]. Through ER stress, triggered signaling pathways will activate the unfolded protein response (UPR) and it is also involved in the regulation of cell energy metabolism, redox status, inflammation, and cell survival [4,5]. The UPR is initiated by the activation of three sensors: protein kinase RNA (PKR-like) ER kinase (PERK), inositol requiring enzyme 1-α (IRE1-α) and activating transcription factor-6 (ATF-6) [6]. Recently, several researches have linked the development of diabetes mellitus, and insulin resistance, to the activation of endoplasmic reticulum stress (ERS) [7,8]. It is known that PERK depletion can lead to diabetes due to the destruction of pancreatic beta cells, probably with the accumulation of C/EBP homologous protein (CHOP) [3,9], while IRE1-α by the activation of X-box binding protein 1 (XBP-1) can protect from insulin resistance [10]. In a normal state these ER sensors are inactivated by the presence glucose regulating protein 78, also known as BIP, a member of the heat shock protein 70 (HSP-70) family. Previous studies have shown that polyunsaturated fatty acids n-3 (ω-3) modulate gene expression of transcription factors such as PPAR and NFXβ, which can help reduce inflammation and insulin resistance (IR) in laboratory animals. However, the effect of this fatty acid in metabolic regulation in patients with IR is still poorly characterized.

Polysaturated fatty acids ω3 eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) can reduce inflammation, but little is known about the effect of ω3 α-linolenic acid (ALA; C18:3n-3). The ALA is the primary fatty acid of the n-3 pathway found in seeds oils, notably those of flaxseeds and chia. It is known that a variable percentage of ALA will be converted to DHA (2%-5%) and EPA (5%-10%) [11]. The consumption of DHA and EPA is approximately 0.1 g-0.2 g per day on average in the North America, while the intake of ALA is 1.4 g per day [12], but the metabolic effects of ALA in situations of insulin resistance still are not well known [13]. Thus, in the present study, we investigated the effects of ALA supplementation on inflammation and ERS in animal models of diabetes mellitus.

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Materials and Methods

Animals and diet

We studied male Wistar rats with 100 g of body weight, obtained from the animal facilities of the School of Medicine of Ribeirão Preto, São Paulo University. The investigation was approved by the local ethics committee on animal research and followed the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication no. 85-23 revised 1996). The animals were maintained on 12 h:12 h artificial light-dark cycles and housed in individual cages. Rats were randomly divided into four groups: control, control+ALA, diabetes and diabetes+ALA. Induction of diabetes was performed by intravenous injection of 40 mg/kg of streptozotocin IV and diabetes mellitus was diagnosed 7 days after, with the demonstration of hyperglycemia (glucose >11 mmol/L). Animals in the control and diabetes groups received a standard rodent chow (3,710 Kcal/Kg; Nuvilab CR1, Sao Paulo, Brazil) ad libitum and the control+ALA and diabetes+ALA groups received the same chow diet supplemented with of 3 g/day of ALA from flaxseed, ad libitum for a period of 8 weeks. Briefly, for the addition of ALA to the diet, 1 L of water as added to 900 g of standard chow, stored over-night at 4°C. After this, 100 g of ALA (Nanomega 3, Henrifarma, Sao Paulo, Brazil) was added and well blended manually, 30 g of supplemented diet was offered every day from 6 pm. There was no restriction of water for all groups. Standard diet composition had 3710 Kcal/Kg, with 62.5% of carbohydrate, 11.8% of lipids and 25.6% of protein and the supplemented diet 4610 Kcal/Kg, 50.3% of carbohydrate, 28.9% of lipids and 20.6% of protein.

After the period of supplementation, the animals were sacrificed by decapitation and after dissection, liver and epidydimal fat were weighed, flash frozen in liquid nitrogen and stored in -70°C until further processing.

Serum glucose, lipids, insulin and cytokines quantification

Blood was collected from tail vein puncture before the supplementation (Pre) and by the end of this period (Post). After centrifugation (400 g) for 10 min in room temperature, plasma was collected and stored in -70°C until assay. Serum glucose was measured by enzymatic method (Glucose HK Liquiform, Labtest, Lagoa Santa, MG, Brazil), and serum cholesterol and triglycerides using Cobas Integra 400 (Roche). Serum cytokines (Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interferon gama (INF-γ)) were measured using Enzyme Linked Immunosorbent Assay (ELISA) Kit (BD Biosciences, New Jersey, USA).

Insulin levels were measured by Ultrasensitive ELISA kit (DRG Diagnostics, Marburg, Germany).

HOMA index was calculated using fasting glucose (mmol/L) and fasting insulin (uU/L) in the following formulas: HOMA-IR=(glucose x insulin)/22.5 and HOMA-β=(20 – insulin)/(glucose -3.5).

Protein extraction and western blot analysis

For protein extraction, we added 1 mL of iced RIPA buffer (30 mM Hapes, 150 Mm NaCl, 10% glycerol, 1% triton X-100, 0.5% sodium deoxicolate and 1 Ml of protease inhibitor per liter) to 100 g of frozen liver tissue and homogenized by a Polytron mixer, under ice-bath. Liver tissue homogenates were incubated for 2 h at 4°C gently shaking. After this period, the homogenates were centrifuged at 12000 rpm for 20 minutes at 4°C and the supernatant transferred to a clean tube. Total protein concentration was determined using the BCA Kit. Protein samples were diluted in sample buffer, heated at 95°C for 10 min and separated by electrophoresis in 10% acrylamide SDS gels. The proteins were transferred to nitrocellulose membranes by semidyry transfer (Trans-blot, Bio Rad) at 25 V for 30 minutes.

For Western blot, the membranes were blocked with TBS-T (1X tris buffered saline + 0.05% tween 20) containing 5% skim milk and then incubated over night with 1:1000 diluted primary antibodies. After washing, the membranes were incubated for 1 hour at room temperature with 1:3000 diluted anti-rabbit antibodies. After the final wash with TBS-T blots were developed using HRP substrate in the ChemiDoc XRS+ System (Bio-Rad). Bands were analyzed using the Image Lab software (Bio-Rad).

Fatty acid quantification

Liver fatty acids were extracted from 500 mg of liver tissue and converted to methyl esters by a direct trans esterification method adapted from Lewis et al. [14] and methyl esters separated on a gas chromatograph (Shimadzu Europe, Duisburg, Germany) equipped with an AOC-20i auto-injector (Shimadzu Europe, Duisburg, Germany) using a fused silica SP-2560 column (100 m, 0.25 mm I.D., film thickness 0.20 μm). Helium was used as carrier gas and make-up gas was air. Synthetic air was used for flame ionization detection at 250°C. Injections were made in the split mode. Fatty acid methyl ester retention times were determined by comparison with external standards (Supelco 37 component FAME Mix; Supelco, Bellefonte, PA, USA). Data is presented as Percentage of fat.

Materials

Nitrocellulose paper (#1620177), Laemmli Sample Buffer (#1610737) and HRP substrate (IMMUN-STAR #1705040) were from Bio Rad Hercules, CA, USA. Bicinchoninic Acid Protein Assay Kit (#BCA1; R9643) Protein Inhibitor Cocktail (#P2714) was from Sigma Chemical Co. (St. Louis, MO). Anti-tumor necrosis factor-alpha (TNF-α) (rabbit polyclonal, SC-8301), anti-heat shock protein 70 (HSP-70) (rabbit polyclonal, SC-71060R), anti-C/EBP homologous protein (CHOP) (rabbit polyclonal, SC-3755), anti-X-box binding protein 1 (XBP-1) (M186) (rabbit polyclonal, SC-7176) and anti-beta actin (β-actin, HRP linked) (rabbit polyclonal sc-1616) antibodies were from Santa Cruz Biotechnology, Inc. Anti-AKT (rabbit polyclonal, #9272S), anti-protein kinase RNA (PKR-like) ER kinase (PERK) (rabbit polyclonal, #C33E10), anti- inositol requiring enzyme 1 alpha (IRE1-α) (rabbit polyclonal, #14C10), anti-heat shock protein 90 (HSP-90) (GRP94) (rabbit polyclonal, #2104S), antiglucagon regulating protein 78 (BIP) (rabbit polyclonal, #C50B12) and anti-rabbit IgG, HRP-linked (goat anti-rabbit, #7074) were from Cell Signaling Technology (Beverly, MA, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis

All numeric results are expressed as the means ± SD or mean ± SEM. The results of blots are presented as direct comparisons of bands. Statistical analysis was performed by employing the ANOVA test with Bonferroni posttest for 3 groups or Student t test when comparing 2 groups. Significance was established at the p<0.05 level.

Results

Food consumption

Animals were fed with chow diet and water ad libitum during the entire period of study. Animals in the ALA supplemented groups...
had the chow diet changed for 30 g of 10% ALA supplemented diet at 6 pm daily. By the morning of the next day the food consumption was checked and the chow diet was returned if there was no more supplemented food. Since the source of omega 3 was not from fish, the diet was well tolerated and consumed entirely in 18 hours after being offered. This ensured a daily intake of 3 g of omega 3 per day extracted from flaxseed. According to the manufacture there is 46% to 52% of ALA in the product, meaning that the daily intake was 1.47 g of ALA in average. The food ingestion by the non-supplemented animals was not systematically measured, but adequate for breed and age.

**Metabolic parameters and weight**

The diabetic groups (diabetes and diabetes+ALA) had increased liver weight compared to control group (p<0.0001), but the epidydimal adipose tissue was smaller only in the diabetes group compared to the control group (p=0.001). Additionally, the diabetes+ALA group had lower liver (p<0.001) and greater epidydimal adipose tissue (p=0.003) weight in relation to the diabetes group (Figures 1A and 1B), besides no difference in total body weight.

There was no difference on the total liver lipid content between the groups (Figure 1C). In our study we observed that the diabetes group had high blood glucose concentration and low serum level of insulin, showing that the dose of streptozotocin injected was able to induce a diabetogenic state, but the ALA supplementation right after the induction of diabetes (24 h) preserved the pancreatic insulin secretion capacity (Table 1). This observation was followed by lower glucose and triglyceride serum levels (p=0.01, p<0.001, respectively) on diabetes+ALA group in post period compared to diabetes group, but no difference in total cholesterol (Table 1). We observed distinct behavior on the cholesterol levels between control and diabetes groups, with a decrease in the first and increase in the second group on post period; however, ALA supplementation decreased the serum cholesterol levels in diabetic animals.

Insulin was measured only in the post period and, interestingly, the diabetes group, as expected, had significantly lower levels compared to the control group, while the diabetes+ALA group did not show any difference to the control group (Table 1).

HOMA-IR index was significantly higher in the control group compared to the diabetes group (1.06 ± 0.26 and 0.29 ± 0.13, respectively), but similar to control+ALA and diabetes+ALA groups (0.97 ± 0.16 and 2.57 ± 2.4, respectively). HOMA-β index was also significant higher in the control groups (control: 34.7 ±23.6 and control+ALA: 54.7 ± 19.0)

![Figure 1](image-url)
compared to diabetes groups (diabetes: 0.5 ± 1.8 and diabetes+ALA: 1.8 ± 2.1) and despite an increase in the ALA supplemented groups, there was no significant difference compared to the non-supplemented. Additionally, serum cholesterol and triglycerides increased in the post period compared to the pre-period in the diabetic group, but no other differences were observed.

**Omega 3 quantification in liver**

We observed that the diabetogenic state determined lower levels of ALA and EPA compared to DHA, while the control groups had lower levels of ALA compared to EPA and DHA and that DHA levels were lower that EPA (Figure 1). There was an increase of 9 and 1.5 folds in ALA levels in the liver of supplemented diabetic and control animals, respectively. The increased levels of arachidonic acid in the diabetic group was significantly reduced with the supplementation, associated to an increase in DHA levels (Table 1). Although, we observed increased levels of total omega 6 and lower levels of omega 3 fatty acids in the diabetic group compared to the control and control+ALA, the supplementation was able to improve the difference in the total omega 3 but not in the omega 6. The supplementation determined a significant increase of ALA levels in diabetes and control groups, and EPA levels only in the diabetic group, while there was no difference in the DHA levels between groups (Table 2).

**Inflammation and ERS assessment**

Serum levels of TNF-α and IL-6 were lower in diabetic animals group compared to control, and ALA supplementation did not determine significant changes. However, the diabetes+ALA group showed a decrease in the interferon-γ (INF γ) serum levels after the supplementation period (Table 2). It was observed also that the protein expression of TNF-α in the liver was higher in the control compared to the diabetes group (p=0.03) and the treatment with ALA supplementation decreased the protein expression of TNF-α, both in controls and in diabetic patients (p=0.006 and p=0.02, respectively) (Figure 2A). The evaluation of proteins related to ERS showed decreased protein expression of BIP, HSP-70 and XBP-1 in the liver of control animals compared to the diabetic animals (Figures 2B-2D). Furthermore, the protein expression of CHOP, in the same tissue, was higher in the control and control+ALA group compared to the diabetic groups (p<0.01 in all comparisons) (Figure 2E). In addition, the protein expression of HSP-90 and IRE1-α was higher in the control animals compared to diabetic animals (p=0.01) (Figures 2F and 2G).

Supplementation with 3 g of ALA determined lower protein expression of BIP, CHOP and IRE1-α (p=0.02, p=0.01, p=0.01, respectively) (Figures 2B,2E and 2G) in the control+ALA compared to control group. In contrast, there was an increase in protein expression of HSP-70 (p=0.003) in the liver of control+ALA animals (Figure 2F). Similarly, ALA supplementation in the diabetic group resulted in lower protein expression of BIP and XBP-1 (P=0.03 and P=0.02, respectively). In diabetic animals, supplementation induced higher protein expression of HSP-70 and HSP-90 (p<0.03 and p<0.01, respectively) and in contrast to that observed in control groups showed increased protein expression of IRE1-α (p=0.02) in supplemented animals. Interestingly, supplementation of ALA was responsible for lower protein expression of AKT in the diabetic group (p=0.03) and a trend towards lower expression in the control group (p=0.08) (Figure 2H).

**Discussion**

In our study we tested the hypothesis that, similarly to EPA and DHA, the ALA has an anti-inflammatory effect leading to attenuation in metabolic abnormalities. Several studies have shown the anti-inflammatory effects of omega 3 in diseases such as rheumatoid arthritis, asthma, Crohn's disease and psoriasis, resulting in amelioration of symptoms [15-18]. Therefore, epidemiological studies show a low prevalence of impaired glucose tolerance and type 2 diabetes in
populations with a high intake of omega 3, as natives of Greenland and Alaska [19-21]. The average intake of 1.4 g of omega 3 daily, when converted to human equivalent dosage [22] would represent 13.8 g of ALA daily, and this amount of ALA can be obtained in 27.6 g of flaxseeds, since the concentration of this nutrient in flaxseeds is around 50% [23]. Our data showed that diabetic animals that received ALA supplementation had decreased blood glucose levels compared to non-supplemented diabetic rats and similar serum insulin levels to control animals, suggesting a reduction in the pancreatic cells injury, as a result of an anti-inflammatory effect. The lower serum triglyceride levels also reinforce the improvement of pancreatic beta cell function. Recent study evaluating the supplementation of EPA and DHA in elderly type 2 diabetic patients found an amelioration in glucose metabolism correlated to an increase in serum EPA concentration [24], also suggesting an improvement in the glucose tolerance.

Streptozotocin has been widely used for induction of diabetes, it is an antineoplastic antibiotic, used for the treatment of pancreatic tumors, with little extra pancreatic toxicity. It is known to be a donor nitric oxide, and after it enters the beta-cell via glucose transporter GLUT2, it can promote the alkylation of DNA and consequently beta-cell death [25,26]. In 2-5 days after streptozotocin injection histological analysis of pancreatic islet shows intense leucocyte infiltration and complete destruction of beta-cell [26,27]. Previous study have shown that EPA and DHA can provide better control of glucose and lipid metabolism, associated to a decrease in oxidative stress [28]. In our study, we observed similar metabolic effect and also lower serum levels of INF-γ after the supplementation period, suggesting an anti-inflammatory effect of ALA in the pancreatic cells. Therefore, our data suggests that ALA protected from the streptozotocin effect on the pancreatic cells, however further studies are needed to confirm this effect. Otherwise, besides no difference in serum levels we did observe lower protein expression of TNF-α in the diabetes and diabetes+ALA liver tissue probably resulting from catabolic state resulting from untreated diabetes.

Additionally, the ALA supplementation reduced TNF-α in liver tissue of control and diabetic animals. Investigating the effect of ALA supplementation in the activation of ERS as a potential mechanism of metabolic diseases, we observed that the diabetogenic state decreased UPR transducer IRE1-α, known to, when activated, promote the mRNA of the XBP-1 cleavage, we evaluated the total XBP-1, which was decreased in diabetic animals supplemented with ALA, suggesting that it had been cleaved, similar to data observed by previous authors [8,10]. However, to our knowledge, we demonstrated for the first time that ALA supplementation can improve IRE1-α pathway in liver.

The heat shock response is part of the UPR and previous studies have demonstrated that HSR can alleviate ERS [29,30], thus we
speculate that the reduction on the IRE1-α/XBP-1 ERS signaling pathway is due to an increase in chaperones, since in our study the chaperones HSP-70 and HSP-90 showed higher protein expression in liver from streptozotocin induced diabetic animals supplemented with ALA. Additionally, the chaperone BIP was decreased with the ALA supplement, both in control and diabetic animals, reinforcing the hypothesis that the ALA supplementation protects from the ERS.

Finally, we have provided novel evidence that ALA supplementation plays a role in insulin resistance by decreasing the protein expression of AKT an important pathway of insulin signaling and resulting in decrease in serum glucose levels. Therefore, the over expression of AKT in animal liver can induce hypoglycemia and hepatomegaly [31]. This study did not aim to determine the mechanisms involved in this regulation, limiting our discussion and raising questions to be answered in future studies. To our knowledge this is the first study that evaluate the effect of the ALA supplementation on the ERS in streptozotocin induced diabetic animals, verifying that effect observed in this study was mediated by an increase in chaperones with preservation of insulin secretion in streptozotocin induced diabetic rats and resulting in lower blood glucose and triglyceride levels associated to a reduction in systemic inflammation.

Conclusion

In conclusion, supplementation of ALA reduced blood glucose and serum triglyceride levels associated to a reduction in systemic inflammation and it was also able to influence important pathways involved in the modulation of ERS.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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