EVALUATION OF EFFECTS OF RESVERATROL ON BRAIN NITRIC OXIDE AND ENERGY METABOLISM IN METABOLIC SYNDROME MODEL

METABOLİK SENDROM MODELİNDE RESVERATROLÜN BEYİN DOKUSU NİTRİK OKSİT VE ENERJİ METABOLİZMASI ÜZERİNE ETKİLERİNİN DEĞERLENDİRİLMESİ

RESVERATROL AND METABOLIC SYNDROME

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Özet

Amaç: Yüksek fruktoz diyeti, beyni nörodejenerasyona ve fonksiyon bozukluğuna duyarlı hale getiren nitrozatif strese yol açmaktadır. Bu çalışmada, fruktozla beslenen sıçanlarda metabolik sendromun (MetS) beyin dokususunda nitrozatif stres ve enerji dengesi üzerindeki etkisinin incelenmesi ve resveratrol uygulamasının muhtemel koruyucu etkilerinin belirlenmesi amaçlanmıştır. Gereç ve Yöntem: Yetişkin erkek Sprague-Dawley sıçanlar kontrol, fruktoz, resveratrol ve fruktoz+resveratrol olmak üzere 4 gruba ayrılmıştır (n=8). MetS %20'lik fruktozun içme suyu içinde verilmesiyle oluşturulmuş, resveratrol uygulaması ise günlük 10 mg/kg dozda oral gavaj yoluyla yapılmıştır. Sistolik kan basınçları (SKB) ölçülmüştür. 8 hafta sonunda, serum trigliserit, glukoz, insülin düzeyleri ile beyin dokusu ATP/ADP oranı, nitrik oksit (NOx) ve 3-nitrotirozin (3-NT) seviyeleri belirlenmiştir. Ayrıca, dokulardaki endotelyal ve indüklenebilir nitrik oksit sentaz (eNOS ve iNOS) protein düzeyleri western blotlama tekniği ile belirlenmiştir. Bulgular: Fuktoz uygulaması, kontrol grubuna göre SKB, serum trigliserit, insülin seviyelerini anlamlı şekilde artırmış ve insülin direncine neden olmuştur. Kontrol grubuyla karşılaştırıldığında, fruktoz doku ATP/ADP oranı ile 3-NT ve NOx düzeylerinde belirgin değişikliğe yol açmamıştır. Resveratrol uygulaması 3-NT ve NOx seviyelerini etkilemezken; resveratrol ve fruktoz+resveratrol gruplarında ATP/ADP oranlarında anlamlı azalmaya neden olmuştur. eNOS ve iNOS proteinleri hiçbir grupta saptanamamıştır. Tartışma: Bulgularımız 8 haftalık yüksek fruktoz diyetinin beyin dokusunda NO üretimi, enerji metabolizması ve protein nitrasyonu üzerinde etkili olmadığını göstermiştir. Tek başına ya da fruktozla birlikte uygulanan resveratrol ise bu dozda pro-oksidan olarak etki etmiştir.

Anahtar Kelimeler

Fruktoz; Resveratrol; ATP; Beyin

Abstract

Aim: A high fructose diet promotes nitrosative stress that makes the brain susceptible to dysfunction and neurodegeneration. The aim of this study was to examine the possible resveratrol effects on brain nitrosative stress and energy balance in the fructose-mediated metabolic syndrome (MetS) model. Material and Method: Adult male Sprague-Dawley rats were separated into four groups (n=8 in each group): control, fructose, resveratrol and fructose plus resveratrol. MetS was induced by fructose solution 20% in tap water, and resveratrol was applied at the dose of 10mg/kg daily by oral gavage. Systolic blood pressures (SBP) were measured by the tail-cuff method. After the experimental period of eight weeks, serum triglycerides, glucose, insulin and total brain tissue ATP/ADP ratio, nitric oxide (NOx) and 3-nitrotyrosine (3-NT) levels were measured. Also tissue endothelial and inducible nitric oxide synthase (eNOS and iNOS) protein levels were determined by western blotting. Results: Fructose increased SBP, serum triglycerides, insulin levels and induced insulin resistance significantly compared to the control group. In comparison with control group, fructose did not cause significant differences in tissue ATP/ADP ratio, 3-NT and NOx levels. While resveratrol had no effect on NOx and 3-NT levels, it caused a decrease in the ATP/ADP ratio in both the resveratrol and resveratrol plus fructose groups. iNOS and eNOS proteins were not detected in any of the groups. Discussion: These results indicate that a high fructose diet for eight weeks did not influence NO production, energy metabolism or protein nitration in rat brain tissues. Nevertheless resveratrol acted as a pro-oxidant at that dose when administered with fructose and alone.

Keywords

Fructose; Resveratrol; ATP; Brain

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Introduction

Metabolic syndrome (MetS) diagnosis implies positive results for at least three metabolic alterations including insulin resistance, hypertension, endothelial dysfunction, hyperinsulinemia and dyslipidemia [1]. It has been reported that high-dietary fructose consumption leads to an increased insulin resistance index, increased insulin and triglyceride levels, and hypertension, which characterize MetS [2]. The involvement of the brain in the pathogenesis of MetS is related to neurochemical changes [3]. However, little is known about the effects of fructose on brain tissue. Some early studies have suggested that fructose can not penetrate the blood-brain barrier in significant amounts. By contrast, accumulating evidence indicates that neuronal cells are able to metabolize fructose, and fructose intake has been shown to disturb the plasma membrane of rat neurons, impairing neuronal function [4]. In addition, it was found that long-term fructose -drinking leads to insulin resistance, impaired insulin signaling, oxidative stress, neuroinflammation, and cognitive impairment [5]. An inflammatory state in the brain regulatory centers, such as the hypothalamus, disrupts its metabolic function and the neuronal and neuroendocrine regulation of a number of physiological processes, such as energy balance, glucose metabolism, and insulin resistance [3,6].

Sustaining the redox homeostasis is essential for the survival of brain cells due to their high metabolic energy requirement to maintain electrochemical gradients, neurotransmitter release, and membrane stability. Brain antioxidant levels are restricted compared to other organs and less able to compensate for reactive and nitrogen species (RONS) generation [7]. Recent studies have indicated the hypothesis that nitric oxide (NO) is a mediator of neuronal injury. It can diffuse freely out of the neurons producing it, reacting with superoxide ($O_2^{\bullet-}$) to form peroxynitrite, its important diagnostic marker, for 3-nitrotyrosine (3-NT), and trigger cell death in the surrounding neurons of the cerebral cortex, cerebellum, and hippocampus [7,8].

Resveratrol (3,4,5-trihydroxystilbene) is present in high concentration in the skin and seeds of grapes. It has several biological effects, including a potent antioxidative effect, antiplatelet, estrogenic, and anti-inflammatory activity [9,10]. Preliminary time-course and dose-response data have revealed that daily administration of various doses of resveratrol to healthy animals for up to seven days is safe and even neuroprotective [11]. Although its mechanism of action is not completely clear, this compound has a strong antioxidant/scavenger activity that protects brain tissues against ischemic damage by interfering with mitochondrial homeostasis [12]. Little is known about the precise effect of resveratrol on the fructose-induced MetS model in brain tissue depending on the nitrosative stress and ATP production. The aim of this study was to examine possible resveratrol effects on brain 3-nitrotyosine (3-NT), nitric oxide (NOx) levels as nitrosative stress markers, and ATP/ADP ratio for energy balance in fructose-fed rats.

Material and Method

Chemicals

Trans-resveratrol (≥99%) was purchased from Cayman Chemical (Spi-Bio, Montigny le Bretonneux, France). D-Fructose (≥99%) was purchased from Sigma-Aldrich (St. Louis MO, USA). Primary and secondary antibodies for western blotting were purchased from Cell Signaling Technology (Danvers, MA, USA).

Animals and Experimental Design

This study was conducted in accordance with the regulations of the Animal Experimentation Ethics Committee of Gazi University (G.Ü.ET-10.037). Thirty-two adult male Sprague-Dawley rats weighing 225±10 g were housed at 20-24°C with a 12-h light/12-h dark cycle and provided with standard rat chow and tap water that was freely available.

Rats were randomly divided into four groups (n=8 in each group) as follows:

1. Control Group: Rats fed a standard rodent diet and tap water.
2. Fructose Group: Rats fed a standard rodent diet and tap water supplemented with 20% fructose [13].
3. Resveratrol Group: Rats fed a standard rodent diet and tap water, and resveratrol administered at the dose of 10 mg/kg body weight in 0.1% ethanol solution per day by oral gavage [14]. Resveratrol solution was prepared freshly every day.
4. Fructose plus Resveratrol Group: Rats fed a standard rodent diet and tap water supplemented with 20% fructose, and resveratrol administered at the dose of 10 mg/kg body weight in 0.1% ethanol solution per day by oral gavage.

Since ethanol was used as the vehicle for resveratrol, the control and fructose groups received 0.1% ethanol solution proportionately with body weight. The experiment was carried out for 8 weeks, at which time, the animals were sacrificed under ketamine (30 mg/kg bw) and xylazine (6 mg/kg bw) anesthesia. Blood samples and brain tissues were taken.

Measurement of Systolic Blood Pressure and Serum Analysis

Systolic blood pressures (SBP) were measured by the tail-cuff method at the beginning of the study, at the end of week 4, and at the end of week 8. Serum glucose and triglyceride levels were measured by enzymatic methods using autoanalyzers. Serum insulin level was estimated by using a commercially available ELISA kit (Millipore, MA, USA). Insulin resistance was determined by the Homeostasis Model Assessment index (HOMA-IR) using the formula: [insulin (mU/L) x glucose (mmol/L)]/22.5.

Measurement of Tissue Nitric Oxide (NOx) and Protein Levels

Tissue NOx (nitrite plus nitrate), which are known to be the end products of NO, was determined by using a commercially available colorimetric kit (Cayman Chemical, Spi-Bio, Montigny le Bretonneux, France). Tissue total protein concentration was evaluated by the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Measurement of Tyrosine Nitration (3-NT) and ATP-ADP Levels

For 3-NT analysis, total brain tissue homogenates were prepared according to the method described by Kamisaki et al. [15]. All samples were analyzed by HPLC with electrochemical detector (ECD) using the method described by Maruyama et al. [16]. Tissue ATP and ADP levels were measured by HPLC diode array detector, using the method described by Szabo et al. [17].

Western Blotting Assay for eNOS and iNOS

Total brain tissues were homogenized with ice cold RIPA buffer. Immunochemical analyses were performed wherein 20 µg protein of brain samples were separated with SDS-PAGE. The separated proteins were then transferred from the gels onto a PVDF membrane, and then blocked. The membranes were incubated with the primary antibodies against either rat eNOS (1:1000) or rat iNOS (1:1000). Then, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (anti-rabbit (1:5000)). iNOS electrophoresis standard and rat aorta tissue were used as positive controls for iNOS and eNOS respectively.

Statistical Analysis

The statistical analyses of the results were calculated using a computerized statistical package (SPSS 16.0 for Windows, Chicago, IL, USA). Each mean value was compared by one-way analysis of variance (ANOVA) and Tukey for multiple comparisons. All statistical tests were two-tailed, and $p<0.05$ was considered statistically significant.

Results

SBP and Serum Parameters

In comparison with the control group, fructose administration caused a significant increase in SBP, serum triglycerides, insulin levels and HOMA-IR (Table 1).

Tissue NOx, eNOS and iNOS Protein Levels

NOx levels did not change significantly among groups (Table 2). In the fructose plus resveratrol group, NOx was higher than in the other groups, but not at a statistically significant level. iNOS and eNOS proteins were not detected in any groups (Figure 1).

Tissue 3-Nitrotyrosine, ATP and ADP Levels

Fructose or resveratrol administration did not cause significant changes in 3-NT levels compared to the control group (Table 2). In the fructose plus resveratrol group, 3-NT levels significantly increased compared to both the fructose and resveratrol groups ($p<0.05$). A significant difference between ATP/ADP ratios of control and fructose groups was not found (Table 3). ATP/ADP ratios were diminished in both the resveratrol and fructose plus resveratrol groups compared with other groups ($p<0.05$). Moreover, the lowest ATP/ADP ratio was observed in the fructose plus resveratrol group.

Table 1. SBP and serum values related to MetS at 8 weeks

	SBP (mmHg)	Triglycerides (mg/dl)	Glucose (mmol/L)	Insulin (mU/L)	HOMA-IR
Control	125.3±1.29	36.00±8.07	12.299±2.19	4.79±1.80	2.67±1.11
Fructose	160.1±1.40[a]	93.75±15.85[a]	13.195±1.54	28.21±6.02[a]	16.71±4.89[a]
Resveratrol	124.3±1.10[b]	55.12±7.49[a,b]	14.236±1.725	8.403±1.238[a,b]	5.342±1.172[a,b]
Fr+Rsv	127.3±2.27[b]	143.62±27.68[a,b,c]	11.294±2.338[c]	34.266±7.334[a,c]	17.021±4.481[a,c]

ap<0.05, compared to control group
bp<0.05, compared to fructose group
cp<0.05, compared to resveratrol group

Table 2. Tissue NOx and 3-NT levels

	NOx (mmol/g tissue)	3-NT (nmol/mg protein)
Control	1.344±0.266	43.136±3.992
Fructose	1.264±0.228	38.905±4.391
Resveratrol	1.262±0.181	38.381±4.258
Fructose+Resveratrol	1.421±0.236	49.173±7.404[b,c]

bp<0.05, compared to fructose group
cp<0.05, compared to resveratrol group

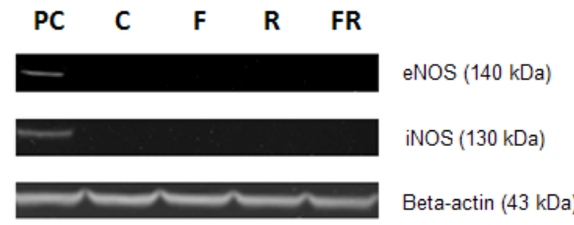

Figure 1. Tissue eNOS and iNOS protein levels
(PC: Positive control, C: Control, F: Fructose, R: Resveratrol, FR: Fructose+Resveratrol)

Table 3. Tissue ATP, ADP levels and ATP/ADP ratio

	ATP (µmol/g tissue)	ADP (µmol/g tissue)	ATP/ADP
Control	0.026±0.003	0.119±0.028	0.228±0.039
Fructose	0.027±0.005	0.120±0.010	0.226±0.037
Resveratrol	0.013±0.001[a,b]	0.094±0.018	0.146±0.025[a,b]
Fructose+Resveratrol	0.013±0.001[a,b]	0.127±0.013	0.103±0.013[a,b,c]

ap<0.05, compared to control group
bp<0.05, compared to fructose group
cp<0.05, compared to resveratrol group

Discussion

Nowadays, it is known that one of the main reasons of increasing incidence of MetS is dietary high fructose consumption. Fructose encourages insulin resistance, glucose intolerance, hypertriglyceridemia, and hypertension in animal models [3]. In our study, we used male Sprague-Dawley rats and fructose administration was accomplished by giving daily prepared 20% D-gructose in tap water for eight weeks. At the end of the study, fructose intake induced the MetS criteria of hypertension, hyperinsulinemia, insulin resistance, and hypertriglyceridemia compared with the control group. Thus, the MetS model was succesfully demonstrated (Table 1).

Data from animal studies show that diet with higher fructose content result in rapid insulin resistance, compensatory hyperinsulinemia and brain abnormalities [18]. It was found that long-term fructose -drinking causes impaired insulin signaling, oxidative stress, neuroinflammation and cognitive impairment in the brain [18-20]. Oxidative/nitrosative stress is reported as one of the earliest events in the pathogenesis of neurodegenerative diseases. Recent studies have supported the hypothesis that nitric oxide (NO) production by neuronal nitric oxide sythase (nNOS) activity is a crucial step

in the pathophysiology of cell death in the brain. NO is a free radical that has several important biological functions. It acts as a vasodilatator and neurotransmitter; in addition, it reacts with O2•- to form peroxynitrite and induce cell death in neurons of the cerebral cortex, hippocampus, and cerebellum [8,21]. Endogenous formation of NO by glutamate receptor activation in cortical neurons leads to rapid and reversible inhibition of mitochondrial ATP synthesis. It has been reported that persistent exposure of mitochondria to NO causes peroxynitrite generation which in turn damages complex-I by nitration of essential tyrosine residue, of which the diagnostic marker, 3-NT [22]. Similarly, diet-induced metabolic disturbances are associated with insufficient formation of ATP, leading to neuropathology [23].

In the present study, brain tissue eNOS and iNOS proteins were analyzed; no detectable level was measured by western blotting in any groups. Fructose drinking for 8 -weeks did not induce the formation of iNOS. Neither was eNOS formation in total brain tissue demonstrated. Our results were consistent with a previous study that NO, formed by nNOS, has a major signaling function in the central and peripheral nervous systems. And nNOs has been thought to account for 95% or more of all NOS catalytic activity in the brain [24].

In our study, as a result of fructose feeding, neither NOx nor 3-NT levels changed significantly, but the systemic symptoms of MetS were induced. Fructose did not effect our parameters, and it did not cause nitrosative stress in the brain. Our results were also consistent with the data from the evaluation of the effect of fructose on oxidative/nitrosative stress of brain carried out by Lopes et al. [4]. They observed NOx levels were not altered during 8-week fructose diet, which may not be sufficient to cause oxidative/nitrosative stress in total brain tissues. In addition, fructose administration did not cause alteration in the ATP/ADP ratio compared with the control group in our study. It is known that high fructose consumption causes cellular ATP depletion, and this situation leads to production of inflammatory proteins, endothelial dysfunction, and oxidative stress [25,26]. In the present study, after feeding fructose, free radical-mediated damage and ATP depletion were not observed. This may be related to the dose and duration of the applied fructose, and the use of total brain tissue may also have been a contributing factor. In recent studies, the hippocampus region of the brain has been shown to be particularly sensitive to high calorie diet such as fructose feeding [27].

To date, resveratrol has been used as a beneficial molecule in clinical and experimental studies. It is accepted to be effective on neurological disorders by attenuating oxidative/nitrosative stress. The beneficial effects are believed to be due to its antioxidative properties [28]. Resveratrol diminishes oxidative stress by directly scavenging free radicals and indirectly increasing endogenous cellular antioxidant defences [29,30]. Further studies suggest that oral 8-week resveratrol administration reduces oxidative DNA damage and also regulates NO levels and eNOS activity, down regulating iNOS activity in rat brains [31]. A previous study has reported that high doses of resveratrol block the insulin signaling, thereby reducing glucose uptake [32]. This compound is able to pass the blood-brain barrier. It exerts neuroprotective effects, upregulates antioxidant enzyme, and is an anti-inflammatory agent [33].

Another important aim of this study was to investigate the effect of resveratrol on NOx, 3-NT levels and ATP/ADP ratio in total brain tissue in the fructose-mediated MetS model. In the present study, resveratrol had no effect on either NOx or 3-NT levels. But when administrated with fructose, in contrast with its previously-observed protective effects, resveratrol increased 3-NT levels, leading to nitrosative stress in the brain. It is unclear how resveratrol increased nitrosative stress in the fructose plus resveratrol group and there has been no report on the cytotoxic property of resveratrol when administrated with fructose. This study may be the first report. Every antioxidant is in fact a redox (reduction–oxidation) agent and thus might become a pro-oxidant to accelerate lipid peroxidation and/or induce DNA damage under special conditions [34]. Resveratrol is known for its antioxidant properties; however, this compound has been proposed to have cytotoxic and pro-oxidant effects depending on its concentration, time of exposure, and cell type [35]. Ahmad et al. reported that resveratrol elicited pro-oxidant properties as evidenced by an increase in intracellular O2•- concentrations in leukemia cells [36]. The pro-oxidant effects of resveratrol were shown on rat liver microsomal systems, and resveratrol increased hydroxyl radical generation [37]. Similarly, Fotiou et al. observed that resveratrol lead to an up-regulation of synaptosomal NO synthase in the rabbit brain, and released NO was converted to peroxynitrite with free oxygen radicals [38]. In our study, when fructose is administered along with resveratrol, it may a effect resveratrol's oxidation/reduction status and thus lead to excess RONS formation, which in turn increases 3-NT levels.

In our study, the ATP/ADP ratio decreased significantly in both the resveratrol and fructose plus resveratrol groups. Mitochondria are a key target of resveratrol and it modulates the mitochondrial respiratory chain function. Complex-I is an important enzyme of respiratory chains and plays a crucial role maintaining mitochondrial homeostasis, not only though its role in ATP production but also in ROS formation. It has been reported that resveratrol binds to the complex-I nucleotide side, which either stimulates or inhibits its activity, according to the resveratrol concentration. At low doses, resveratrol may act as an antioxidant, stimulating the cellular proliferation and the antioxidant response, while at higher concentrations, it may become a pro-oxidant molecule, including cellular damage and decreasing ATP production in the brain [39]. In the present study, decreases in ATP/ADP ratio in both the resveratrol and fructose plus resveratrol groups may be related to resveratrol's dose and its antagonistic effect with fructose.

Our results indicate that fructose consumption at the administered dose and duration did not influence NO production, protein nitration, or ATP/ADP ratio in brain tissue. Resveratrol both alone and together with fructose caused ATP depletion and increased 3-NT levels in brain tissue, thus playing a pro-oxidant role in our experimental conditions. Further experimental studies (e.g., different resveratrol' doses, nNOS analysis and/or investigation of distinct brain areas particularly) are needed to clarify the underlying mechanism of resveratrol on disturbances of these parameters in fructose-fed rats.

Ethical Responsibilities

All institutional and national guidelines for the care and use of laboratory animals were followed.

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Competing Interests

The authors declare that they have no competing interests.

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