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Research Article

Determining Gene Expression Profile of GPX 1 in the Liver of Diabetic Rats Treated with Capsaicin by Real-Time PCR [1]

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Abstract

The purpose of the present study was to determine the Glutathione Peroxidase 1 (GPX 1) gene expression by Real Time PCR in the liver of healthy and diabetic rats treated with capsaicin. Twenty Sprague-Dawley rats were used in our study. Rats were divided into four groups: Group I: diabetic rats (n=5), Group II: capsaicin injected rats (n=5), Group III: Capsaicin injected diabetic rats (n=5), and Group IV: control rats (n=5). Capsaicin injection began 72 h after streptozotocin (STZ) injection. Capsaicin (1 mg/kg) was prepared in 10% ethanol, 1% Tween 20, and 80% sterile water and subcutaneously injected daily in both Groups II and III for two weeks. The results of RT-PCR conducted to determine GPX 1 gene expression indicated that capsaicin treated diabetic rats had higher GPX 1 expression compared to all groups including control ($P<0.05$). As a result, capsaicin causes an increase in GPX 1 gene expression at transcription level in the diabetic rat liver. Further investigation is needed to determine if such an increase occurs at protein level using various methods such as western-blot analysis.

Keywords: *GPX 1, RT-PCR, Capsaicin, Diabetes, Liver, Rat*

Kapsaisin Uygulanan Diabetik Sıçanların Karaciğer Dokusunda GPX 1'in Real Time-PCR ile Expresyon Profilinin Çıkarılması

Özet

Bu çalışmada, Kapsaisin (CAP) uygulanan, sağlıklı ve diabetik sıçanların karaciğer dokusunda Glutatyon Peroksidaz 1'in (GPX 1) RT-PCR ile belirlenmesi amaçlanmıştır. Araştırmada 20 adet Sprague-Dawley ırkı sıçan, Diyabet (Grup I), Sadece Kapsaisin Uygulanan (Grup II), Kapsaisin uygulanmış Diabetli (Grup III) ve Kontrol (Grup IV) olmak üzere dört gruba ayrıldı. Kapsaisin uygulamasına, streptozotosin (STZ) enjeksiyonundan 72 saat sonra başlanıp, 2 hafta boyunca hem diabetik gruba hem de sadece kapsaisin uygulanan gruba her gün 1 mg/kg kapsaisin, %10 ethanol, %1 Tween 20 ve %80 distile su ile çözdürüldükten sonra subkutan olarak uygulandı. Karaciğerdeki GPX 1 geninin ekspresyonu için yapılan RT-PCR analizi sonucunda; kapsaisin uygulanan diabet grubunda GPX 1'in gen ekspresyonunun kontrol grubu da dahil diğer gruplara göre daha yüksek olduğunu belirlendi ($P<0.05$). Sonuç olarak diabette kullanılan kapsaisinin GPX 1 transkripsiyon artışına neden olduğu ancak protein seviyesinde bir artışa yansıyıp yansımadığının, western-blot gibi metodlarla da belirlenmesi gerektiği sonucuna varıldı.

Anahtar sözcükler: *GPX 1, RT-PCR, Kapsaisin, Diabet, Karaciğer, Sıçan*

INTRODUCTION

Diabetes mellitus is a metabolic disorder with three metabolic types that occurs due to insulin resistance or, rarely, deficient insulin secretion and is characterized by hyperglycemia [1,2]. Type I or the juvenile type develops as a result of autoimmune inflammation against pancreatic β cells while Type II occurs later in life, secondary to insulin resistance. Type III, also called the gestational diabetes, only occurs during pregnancy and is due to insulin resistance, or rarely to β cell failure. Oxidative stress has been implicated in both the pathogenesis and complications of three types of diabetes [1]. Under normal circumstances, the rates of formation and clearance of free radicals are in a fine

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equilibrium, which is referred to as oxidative equilibrium. An increase in the rate of formation or a decrease in the rate of clearance of free radicals is called oxidative stress [3,4]. In parallel to increasing oxidative stress, the amount of free radicals also increases [5]. The latter event causes lipid peroxidation, DNA damage, and inactivation of multiple enzymes [4]. While low levels of reactive oxygen species play a role in intracellular signaling pathways involving cell differentiation, cell progression, or halt of cell growth and apoptosis, their increasing doses such as those in oxidative stress causes metabolic disorders and damage to biological macromolecules [6]. Former studies have shown that diabetes leads to oxidative stress by lowering the antioxidant potential and increasing free radical formation rate [7]. It has been reported that hyperglycemia particularly induces oxidative stress in the cell [8,9]. Oxidative stress emerges not only in diabetes, but also in certain other pathological conditions such as cardiovascular diseases, aging, cancer, and neurological disorders [7]. In human body, there are plenty of endogenous and exogenous defensive mechanisms collectively called antioxidants to prevent formation of free radicals and neutralize their detrimental effects. Such mechanisms can be grouped as agents that prevent free radical formation or neutralize already formed free radicals; they may also be classified as enzymes and non-enzymes [10]. Glutathione peroxidase (GPX), which has been reported to exist in mitochondria and cytosol [11], is an endogenous enzyme [10], which is responsible from degradation of hydroperoxides [10,11]. It has been formerly reported that, depending on the tissue types, it has at least 5 isoforms in mammalian cells and GPX 1 is especially abundant in erythrocytes, kidneys, and liver [12]. As a chronic metabolic disorder, diabetes is characterized by increased oxidative stress. As a result, increased free radicals engage an interaction with nucleic acids, proteins, and lipids, ultimately causing loss of membrane integrity, functional and structural alterations in proteins, and genetic mutations [13]. Diabetes-induced oxidative stress renders liver and other tissues more susceptible to various complications [14]. Increased lipid peroxidation and reduced glutathione levels are characteristic in diabetes [15]. Moreover, oxidative stress plays an important role in eliciting diabetic complications and underlies its pathogenesis [16]. Majority of the data suggesting a role of oxidative stress in initiating diabetes comes from animal studies that have employed alloxan and streptozotocin (STZ) to induce diabetes [10]. In this study STZ an agent that destroy β cells in pancreas, by inhibiting N-Acetyl-β-D-Glucosaminidase enzyme, was used in order to induce diabetes [17].

Capsaicin, which was used in the study, is the active ingredient of hot pepper [18,19]. It has been suggested that capsaicin has certain effects on gastrointestinal, cardiovascular, respiratory, limbic, and thermoregulatory systems [20]. Particularly used for arthritis management, capsaicin inhibits superoxide anion formation and changes the redox state of the cell [18]. Capsaicin metabolism is similar in human, dog, and rat and it is rapidly metabolized by hepatic enzymes. In addition to the main metabolites in these species, namely 16-hydroxycapsaicin, 17- hydroxycapsaicin, and 16,17- dehydrocapsaicin; microsomes and S9 fractions in rats also produce vanillylamine and vanillic acid. It has been reported that capsaicin is activated in liver by mix-function oxidase systems and turned into an electrophilic intermediary substance, which is able to covalently bind to hepatic proteins [21]. It has been observed that capsaicin has *in vitro* regulatory functions on cellular growth and collagenase and prostaglandin synthesis in rheumatoid arthritis. It also regulates lymphocyte proliferation, antibody production, and neutrophil chemotaxis [18]. Capsaicin has been shown to be effective in diabetic neuropathies [22]. In a study performed in rats, it has been observed that capsaicin induced lipid mobilization in fatty tissue and lowered triglyceride levels in serum [23] and liver [18]. As a regulatory molecule having certain effects on fat and energy metabolism, capsaicin has also been reported to possess some anti-obesity properties by lowering the blood fat content and inhibiting proliferation of the white fat cells [24]. Furthermore, it has been suggested that capsaicin has oxidative stress lowering effects by increasing the levels of antioxidant molecules and enzymes especially in liver and erythrocytes [25].

In the present study, it was aimed to determine the mRNA expression of Glutathion Peroxidase 1 (GPX 1), an antioxidant enzyme, in liver tissues of capsaicin administered healthy rats and rats with STZ-induced experimental diabetes.

MATERIAL and METHODS

Experimental Animals

This study was conducted after obtaining the approval from Kirikkale University Animal Experiments Local Ethics Committee (No:23.02.2012/12). It enrolled 20 female Sprague-Dawley rats with an average age of 8-12 weeks. The rats were housed in standard cages with alternating 12-h light-dark cycles at a temperature of 22±2°C and an average humidity of 50±5%. They were fed ad libitum with standard rat feed and water. The rats were grouped into 4 groups each containing 5 rats. Group I: STZ Diabetes Group (n=5); Group II: Capsaicin only Group (n=5); Group III: Capsaicin administered STZ Diabetes Group (n=5); Group IV: Control Group (n=5). Group I and Group III were administered STZ (Sigma, St Louis, MO, USA) dissolved in fresh citrate tampon (pH 4.5; 0.1 M) via intraperitoneal (IP) route in a single dose of 45 mg/kg [26]. Then, a blood sample was obtained from the tail veins of the animals following an 8-h fasting period 72 h after STZ injection and rats having a blood glucose level of 200 mg/dl or higher measured by a hand glucometer (Accu-Chek-Go, Roche, Switzerland) were considered diabetic [27] and included in the study.

After the third day, when diabetes was confirmed, Group II and Group III were subcutaneously injected with capsaicin 1 mg/kg (Sigma, St Louis, MO, USA) (dissolved in 10% ethanol, 1% Tween 20, and 80% distilled water) every day for 2 weeks [28].

Tissue Sampling

Liver tissue samples were taken at 14th day after sacrifice of rats with cervical dislocation under ether anesthesia. Liver tissues harvested for molecular analysis were homogenized in Tri-Reagent (Sigma, St Louis, MO, USA) and stored at +4°C until the day of analysis.

RNA Isolation and c-DNA Synthesis

Total RNA isolation was performed using the Tri-Reagent (Sigma, St. Louis, MO, USA) obtained by modification of the guanidine isothiocyanate/Phenol-chloroform method described by Chomczynski and Sacci [29]. RNA concentration per microliter was measured at a wavelength of 260 nm. From each total RNA, a 4 µg sample was taken and cDNAs were obtained using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). The cDNAs were then stored at -20°C to be later used in real time PCR.

RT-PCR Analysis

Gene expression was carried out using Real-time PCR. Each PCR reaction contained 3 µl water, 1 µl forward primer (5'CAGTTCGGACATCAGGAGAAT3'), 1 µl reverse primer (5'AGAGCGGGTGAGCCTTCT 3') [30] and 10 µl SYBR Green Supermix (Roche) and 5 µl cDNA to a total volume of 20 ml. mRNA quantification was performed in LightCycler 480 instrument by using SYBR Green I reagents (Roche diagnostics, USA) PCR reaction was induced as 5 min in 95°C, 45 cycles in 10 sec at 95°C. Each analysis was conducted with 5 biological repeats and 3 technical repeats. Glyceraldehyde 3 Phosphate Dehydrogenase (GAPDH) was used for normalization of GPX I gene expression. Forward 5'ACCACAGTCCATGCCATCAC3' and reverse 5'TCCACCACC CTGTTGCTGTA 3' [31] primers were used for GAPDH gene expression. Normalization of gene expression was performed as described by Kayan et al.[32]. Normalization was done with the Delta Ct method ($\Delta Ct = Ct_{target\ gene} - Ct_{housekeeping\ gene}$).

Statistical Analysis

The arithmetical means of the technical repeats was compared with the SPSS software package using the t-test.

RESULTS

Examination of the gene expression in the samples revealed that only the capsaicin-administered diabetes group demonstrated a significant difference *(Table 1, Fig. 1)*. Compared with the control group, the capsaicin only group and the diabetic group had no significant differences in GPX I expression. In the capsaicin-administered diabetes group, on the other hand, GPX I's gene expression was found significantly higher compared to the other groups ($P<0.05$)

DISCUSSION

Under normal circumstances, the rate of production of reactive oxygen species (ROS) and the antioxidant defense system are in a fine equilibrium; an imbalance in favor of ROS results in oxidative stress [4]. There are enzymatic and non-enzymatic cellular antioxidant defense mechanisms to reduce the detrimental effects of ROS [33]. The main enzymes of the antioxidant defense mechanism, namely

Table 1. *Ct levels of the groups*
Tablo 1. *Grupların Ct değerleri*

Groups	Control	Capsaicin	Diabetes	Diabetes + Capsaicin
Ct Levels	9.07	8.58	9.79	4.56*

** P<0.05, n=5*

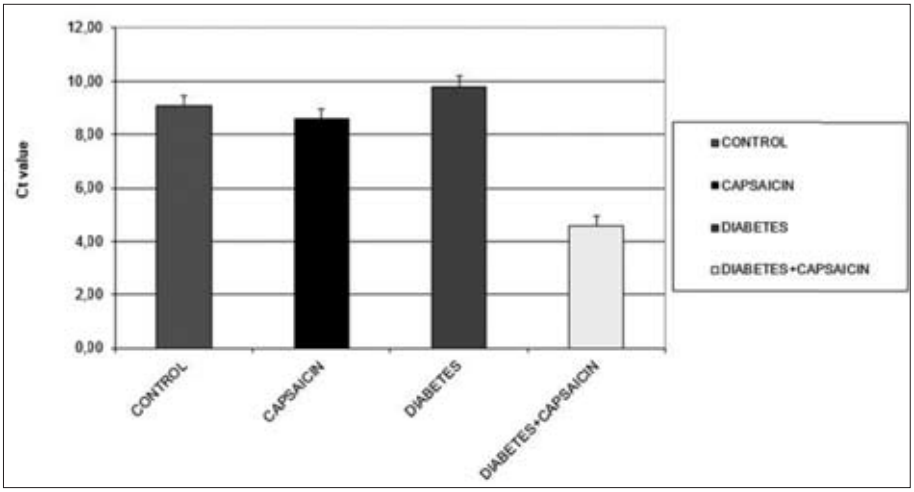

Fig 1. Ct levels of the groups (diabetes + capsaicin),* P<0.05, n=5

Şekil 1. Gruplara ait Ct değerleri (diabet + kapsaisin), * P<0.05, n=5

superoxide dismutase (SOD), glutathione peroxidase, and catalase are overwhelmed by excessive expression of ROS or chronic hyperglycemia. As a result, a vicious cycle is created in which ROS and RNS (Reactive nitrogen species) are incrementally produced and the oxidative stress pathways are activated [34].

Glutathione peroxidase (GPX), an enzymatic antioxidant [33], reportedly possess 5 isoforms in mammals and its levels vary by tissue type [6]. It has in vivo protective action against reactive oxygen species and is the first selenoprotein described in mammals [1]. It has cytosolic and mitochondrial forms and it reduces hydroperoxides of fatty acids [6]. Having been reported to possess regulatory functions on apoptotic signal pathways in various cells and tissues, GPX I has been implicated in the pathogenesis of many disease states including diabetes [1]. Studies on mice have demonstrated that GPX I overexpression strengthens cells against oxidative stress, while its absence promotes susceptibility to oxidative stress [11].

Liver is the main organ for free radical reactions, oxidation and detoxification processes. Therefore, biomarkers of oxidative stress are found elevated at early stage of many disorders [35]. It has been reported that the activity of many antioxidants are reduced [14,34], leading to increased oxidative stress in diabetes [34]. On the other hand, there are also some studies suggesting increased activity of antioxidant system [4,15]. Moreover, conflicting data have been reported by different studies on GPX expression during oxidative stress in rats with experimentally formed diabetes. For instance, both decreased [16,36] and increased [37,38] GPX enzyme expression compared to controls have been reported by separate studies in rats with STZ-induced diabetes. On the other hand, it has also been reported that, when compared with the controls, no significant difference was observed for GPX at hepatic mRNA level [39]. This study also observed that there was no significant difference between diabetic rats and the controls with respect to GPX expression. Such different results obtained in GPX expression of diabetic rats was attributed to various experimental conditions such as age and race of the rats and the duration of the experiment [16].

In the present study, no difference was found in the capsaicin only group compared to the control group with regard to GPX expression. Additionally, GPX I gene expression was found quite elevated in the capsaicin administered diabetes group compared to the controls. This was attributed both to capsaicin's inhibitory effects on oxidative stress [25] and, partially, free radical formation [40] by augmenting antioxidant molecules and enzymes, and also to its antioxidant actions originating from its phenolic OH groups [41] and its stimulant effect on antioxidant defense system [40].

In conclusion, this study suggests that capsaicin has a potential protective effect against hepatic oxidative stress in diabetes via triggering GPX I gene expression. It can be suggested that it needs to be verified by certain methods such as Western-Blot that that increase in transcription is reflected as an augmentation in protein level.

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