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Effect of ß- Caryophyllene on Urocortin-3 expression in adipose tissue of high fat diet and fructose-induced type-2 diabetic rats.

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Abstract:

Urocortin 3 (Ucn 3), a member of the Corticotrophin-releasing Factor (CRF) family of peptides, is strongly expressed in mammalian brain, skeletal muscle, adipose tissue, and pancreatic β cells and has been shown to stimulate insulin secretion. The purpose of this study was to determine the expression of UCN3 levels in high-fat and fructose-induced type-2 diabetic rats' visceral adipose tissue (VAT), the relationship between UCN3 levels and insulin resistance, and the effect of β-caryophyllene on UCN3 expression in high-fat and fructose-induced type-2 diabetic rats. Diabetic rats were generated by giving rats a high-calorie food composition with 2% cholesterol, 1% cholic acid, 30% coconut oil, 67% regular rat feed, and 25% fructose through drinking water for 9 weeks. Then the rats were treated with an oral effective dose of 200 mg of β-caryophyllene or 50 mg of quercetin (QCT)/kg b. wt. once a day for 30 days to find out whether ß-caryophyllene regulates URC3 expression. The data shows that ß-caryophyllene treatment significantly decreases the mRNA and protein expression of urocortin-3 in diabetic rats, the same as the standard drug quercetin. β- Caryophyllene reduces the expression of urocortin-3 and the risk of insulin resistance in type 2 diabetes by reducing inflammation brought on by oxidative stress through β-caryophyllene's antioxidant activity.

Keywords:

β-caryophyllene (BCP), Urocortin 3 (Ucn3), URC3 gene expression.

Inrtoduction:

The corticotropin-releasing factor (CRF) family, which includes four recognised peptide hormones CRF and three urocortins (UCN 1-3) and two G protein-coupledreceptors CRFR1 and CRFR2, is a well-established neuroendocrine signaling peptide that regulates physiological responses to stress via the hypothalamic- pituitary-adrenal (HPA) axis [1]. CRF peptides were discovered in the brain first, butthey are also found in peripheral metabolic tissues such as skeletal muscle, adiposetissue, and the pancreas[2]. The central and peripheral nerve systems, both of which are implicated in the neuroendocrine framework, are known to play a substantial role in stress's effects on metabolic function and the emergence of metabolic disorders[3]. Stress in both the physical and psychological realms is a major [4].Although the CRF system is not fully understood, altering it has been suggested as a treatment for issues with human metabolism. UCN3, which is highly abundant in the pancreas, is thought to protect against hyperglycemia brought on by high-fat diets while simultaneously elevating energy expenditure[5]. Although the expression of UCN3 in tissues that are resistant to insulin, such as adipose tissue, is unknown in high-fat diet-induced type-2 diabetes. Adipose tissue is an endocrine organ that has an impact on both glucose and lipid metabolism [6]. Previous research has shown that insulin resistance in adipose tissue is one of the pathophysiological pathways involved in the development of type- 2 diabetes[7]. As a result, UCN3 may be a therapeutic target for metabolic illness management. The location of UCN3 in adipose tissue, as well as the mechanisms underpinning its participation in adipose tissue-related insulin resistance, are still unknown. Despite its importance in energy balance and insulin production, the circulating and adipose tissue levels of UCN3 in high-fat and fructose-induced type-2 diabetes obesity have never been described. As a result, the following were studied in this study: (1) the expression of UCN3 levels in high-fat and fructose-induced type-2 diabetic rats' visceral adipose tissue (VAT);

(2) the relationship between UCN3 levels and insulin resistance; and (3) the effect of β-caryophyllene on UCN3 expression in high-fat and fructose-induced type-2 diabetic rats.

Materials and Methods

Chemicals

The Sisco Research Laboratories in Chennai, India, and the Sigma-Aldrich Chemical Company in St. Louis, Missouri, the United States; Eurofins Genomics India Pvt Ltd (Bangalore, India); New England Biolabs (NEB) (USA); Promega (USA); Santa Cruz Biotechnology (USA) and Cell Signaling Technology (USA). β-actin monoclonal antibody was bought from Sigma (USA). Total RNA isolation reagent (TRIR) was obtained from Invitrogen, USA. The reverse-transcriptase enzyme was boughtfrom New England Biolabs (NEB) (USA) and Go Taq Green

master mix was obtained from Promega (USA). Urocortin-3 and β-actin primers were purchased from Eurofins Genomics India Pvt Ltd Bangalore, India] and Polyclonal Urocortin-3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, C.A). Provided the chemicals, reagents, and Quercetin used in this experiment. All of these materials were of the molecular and analytical quality. Tokyo Chemicals Industry Co., LTD of Tokyo, Japan was the manufacturer and supplier of β-caryophyllene. Additionally, ACON Laboratories, Inc. sold blood glucose test strips in San Diego, California, USA.

Animals

The experimental investigation was approved by the Institutional Animal Ethics Committee in accordance with the National Guidelines and Protocols and registered with registration number 765/03/ca/CPCSEA and approval certificate number 007/2019 dated April 11, 2019. At the Meenakshi Medical College and Research Institute, the Central Facility for Caring Animal Unit collected and cared for healthy adult male Wistar albino rats (150–180 days old, weighing 180–200g). They were given a regular rat pellet meal provided by Lipton India, Mumbai, (India), and free access to clean drinking water was provided.

Induction of Type-2 Diabetes

By giving rats a high-calorie food composition with 2% cholesterol, 1% cholic acid, 30% coconut oil, 67% regular rat feed, and 25% fructose through drinking water for 9 weeks, type 2 diabetes was generated in the rats according to Nampurath et al., 2008 technique[8]. After nine weeks, animals were chosen for the study if their fasting blood glucose levels were higher than 120 mg/dl. The study's conclusion saw a continuation of the high-fat diet and sugar feeding. The control rats were fed regular pelleted rat food and given unlimited access to water.

Experimental design

The following experimental design was framed, and accordingly the rats were subjected to treatment for a period of one month. Healthy adult male Wistar rats were divided into the following groups of 6 rats each.

Group I: Control (Normal rats).

Group II: Rats were made diabetic (type-2) after feeding high fat diet & fructose through drinking water (30%) for 60 days.

Group III: Type-2 diabetic rats treated orally with β-caryophyllene (200 mg/kg b.wt/day) for 30 days

Group IV: Type-2 diabetic rats treated orally with Quercetin[9] (50 mg/kg, b.wt/ day) for 30days

Group V: Control rats administered orally with β-caryophyllene (200 mg/kg b.wt/day) for 30 days.

The drugs were administrated orally by using 18 gauge ball tipped gavage needle for 30days, The animals were fasted overnight Physiological saline was injected into the anaesthetized animals after sodium thiopentone (40 mg/kg b.w.t.) was administered intraperitoneally to anaesthetize them. And the visceral adipose tissue was cut out to assess various qualities. Blood was then collected.

mRNA expression analysis

Total RNA Isolation, cDNA conversion and real-time PCR

A TRIR kit (Total RNA Isolation Reagent Invitrogen) was used to extract total RNA from the control and experimental samples. In a nutshell, 100 mg of fresh tissue received 1 ml of TRIR, which was then homogenised. The material was then immediately transferred to a micro centrifuge tube, combined with 0.2 ml of chloroform, vortexed for 1 minute, and stored at 4°C for 5 minutes. Then, the mixture was centrifuged at 12,000 g for 15 minutes at 4 °C. Carefully transferring the top layer of the aqueous phase to a fresh microfuge tube, equal parts of isopropyl alcohol were then added, vortex for 15 seconds, and then placed on ice for 10 minutes. Following centrifugation of the material at 12000g for 10 minutes at 4C, the supernatant was separated. The RNA pellet was washed in 1 ml of 75% ethanol using the vortex. The extracted RNA was calculated using spectrometry according to Fourney et al[10]. Each sample's RNA content was quantified in micrograms.

Using a reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was created from 2 micrograms of total RNA in accordance with the manufacturer's instructions. A 45 µl reaction mixture containing 2x reaction buffer (Takara SyBr green master mix), forward and reverse primers for the target and housekeeping genes, water, and β-actin primer sequences are supplied in (Table:1) was made in order to perform real-time PCR. About 5 µl of control DNA for the positive control, 5 µl of water for the negative control, and 5 µl of template cDNA for the samples were extracted and added to each individual PCR vial along with the reaction mixture (45 µl). The reaction was set up for 40 cycles (95°C for 5 min, 95°C for 5 s, 60°C for 20 s, and 72°C for 40s), and the PCR machine (Stratagene MX 3000P, Agilent Technologies, 530l, Stevens Creek Blvd, Santa Clara, CA, 95051) showed the findings on a graph. From the examination of the melt and amplification curves, relative quantification was derived.

Table: 1 Primer sequences of Urocortin molecules

Name of the gene	Primer Sequence	Reference
Ucn3[11]	Sense primer: 5‟- CGAAGTCCCTCTCACACCTGGTT -3‟ Anti-sense primer: 5‟- CGGCAAACGGACAGAAGCATT -3‟	Deyana et al, 2021
Rat β-actin[12]	Sense primer: 5‟- AAG TCC CTC ACC CTC CCA AAA G-3,, Anti-sense primer: 5‟- AAG CAA TGC TGT CAC CTT CCC-3'	Peinnequin et al, 2004

Protein expression analysis

Protein isolation and western blotting

100 mg of adipose tissue from control and experimental animals were used to isolate proteins. 1 ml of buffer A (5 mM NaN3, 0.25 M sucrose, 10 mM NaHCO3) was added to 100 mg of adipose tissue, homogenised, and centrifuged at 1300xg at 4°C for 10 minutes. The supernatant was separated and centrifuged at 12,000xg for 15 minutes at 4°C. To evaluate the Urocortin-3 molecules, the final supernatant was sampled as a total protein. The protein estimation was done using the Lowry et al.[13] technique.

The lysate proteins (50g/lane) were isolated and electro blotted onto a polyvinylidene difluoride (PVDF) membrane Bio-Rad Laboratories Inc] using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 % gel). The membranes were blocked with 5% non-fat dry milk and tagged with primary antibodies (1:1000 dilutions). After three washes with TBS-T, the membrane was incubated for 1 hour with a 1:5000 dilution of horseradish peroxidase-conjugated rabbit-anti-mouse or goat-anti-rabbit secondary antibody (GeNei, Bangalore, India). Following the incubation period, the membrane was washed three times with TBS and TBS- T. The protein bands were visualised using a sophisticated Chemiluminescence detection system (Thermo Fisher Scientific Inc., Waltham, MA, USA), the specific signals were found, and protein bands were captured and quantified using Chemidoc and Quantity One image analysis systems from Bio-Rad Laboratories, CA. The membrane was then stripped for 30 minutes at 50°C in stripping buffer (50 ml, 62.5 mMTris–HCl (pH 6.7), 1 g SDS, and 0.34 ml –mercaptoethanol). The membranes were then re-probed using an anti β -actin antibody (1:5000). The invariant control used was β - actin.

Statistical analysis

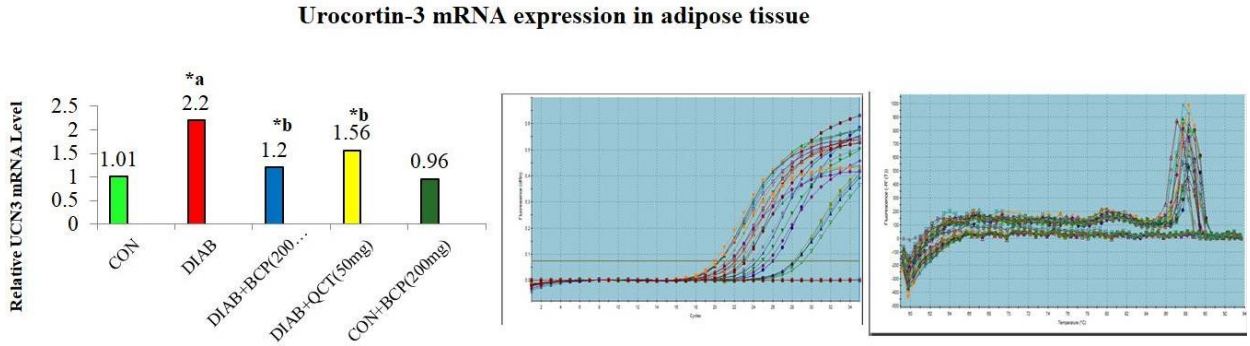
Using one-way analysis of variance (ANOVA) and Duncan's multiple range test, computer-based software, the data were analyzed to determine the significance of individual variance within the control and treated groups (Graph Pad Prism version 5). Duncan's test was used to determine significance at the level of $p<0.05$.

Result:

β- Caryophyllene modulates adipose tissue Urocortin-3 expression in type-2 diabetic adult rats.

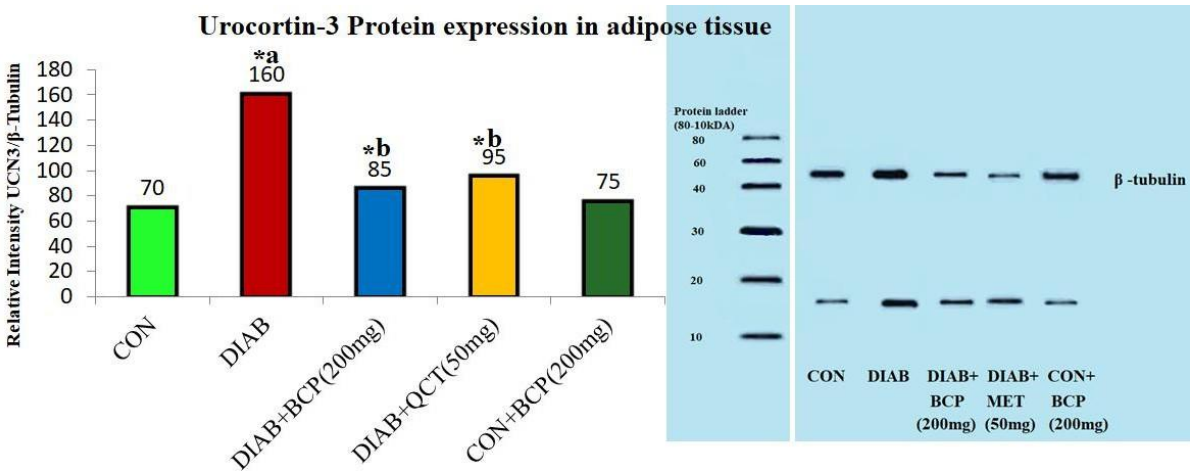
In the current investigation, it was discovered that high-fat and fructose-induced type-2 diabetic rats had elevated levels of UCN3 mRNA and protein in their adipose tissue. However, diabetic rats treated with ß-caryophyllene had considerably improved UCN3 mRNA and protein levels. According to studies, increasing corticosterone caused by stress increases UCN3 gene expression 12], which in turn causes a rise in urocortin-3 protein in tissues that respond to insulin. By reducing lipid peroxidation in type-2 diabetic rats, β-Caryophyllene, a strong antioxidant and anti-inflammatory drug 13], significantly boosts the antioxidant potential.

Figure.1-: Effect of β-caryophyllene on Urocortin-3 mRNA expression in adipose tissue of high fat diet and fructose induced type-2 diabetic rats.



Each bar represents mean ± SEM of 6 animals. Significance at p< 0.05, **a**-compared with control, **b**- compared with diabetic control**.**

Figure.2-: Effect of β-caryophyllene on Urocortin-3 protein expression in adipose tissue of high fat diet and fructose induced type-2 diabetic rats.



Each bar represents mean ± SEM of 6 animals. Significance at $p< 0.05$, **a**-compared with control, **b**- compared with diabetic control**.**

Discussion:

We have previously reported that feeding Wistar rats a diet supplemented with high fat and fructose for 8–9 weeks causes insulin resistance in the animals[14]. By reducing lipotoxicity and maintaining antioxidant capacity without altering caloric intake, 200 mg/day of β- caryophyllene significantly and equally reduces HFD-induced insulin resistance[15]. Here, we show that these observed increases in HFFD-induced type-2 diabetic adipose tissue Urocortin-3 expression. Additionally, quercetin and β-caryophyllene supplementation reduce the expression of Urocortin-3 in visceral white adipose tissue. Increases in visceral adipose tissue Urocortin-3 have been linked to adverse metabolic effects, such as insulin resistance and type-2 diabetes[16], and it has been demonstrated by numerous researchers that supplementing with β-caryophyllene reduces insulin resistance[17]. In addition to tissue bulk, it's also probable that the physiology of adipose tissue plays a significant role in the metabolic dysfunction brought on by the inflammation-oxidative stress combination[18]. In line with this theory, Pathak MP et al. (2021) found that β- caryophyllene supplementation improved pro-inflammatory adipokines from visceral white adipose tissue, followed by generation of oxidative stress in the respiratory system[19]. In a prior work, we found that supplementing with a high-fat diet causes oxidative stress whereas supplementing with β-caryophyllene reduces oxidative stress[20]. Insulin resistance in adipose tissue is caused by oxidative damage and pro-inflammatory adipokines. Additionally, urocortin-3 expression is induced by stress-mediated inflammation. In the current investigation, oxidative stress and inflammation led to increased expression of urocortin-3 in adipose tissue. It

was backed up by rising adipose tissue inflammation, which Pathak MP et al. and our previous work also demonstrated, and rising oxidative stress. These two causes lead to an increase in the urocortin-3 expression that we have seen in adipose tissue.

Conclusion:

The gathered information demonstrates that urocortin-3 expression in adipose tissue is induced by high-fat and fructose dietary supplements. Treatment with β-caryophyllene reduces the expression of urocortin-3 and the risk of insulin resistance in type-2 diabetes by reducing inflammation brought on by oxidative stress through β-caryophyllene's antioxidant activity, similar to the conventional medication quercetin. More research is required to fully understand the β-caryophyllene mechanism of action in urocortin-3-mediated insulin resistance in type 2 diabetes.

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