Piperine Regulates Inflammatory Signalling Molecules in Adipose Tissue: A High Fat Diet Sucrose Induced Type II Diabetic Experimental Model

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Abstract

Metabolic inflammation is an essential event in obesity-induced diabetes and insulin resistance. An extract of the active phenolic component of black pepper (Piper nigrum), piperine, is well known to provide beneficial physiological effects. This study aims to assess the regulation of inflammatory signalling molecules in adipose tissue by Piperine using a high diet and sucrose-induced type II diabetic experimental model. Our results suggest that piperine has the potential as a therapeutic drug or dietary supplement.

Keywords: Control Group, Fasting Blood Glucose, Piperine, Polymerase Chain Reaction.

Introduction

Obesity and type 2 diabetes mellitus (T2DM) have been a great threat worldwide in recent years. According to the Global Diabetes Atlas (9th edition, International Diabetes Federation), the global incidence of diabetes was 9.3%, and the total number of patients was 463 million in 2019. By 2045, these two figures will increase to 10.9% and 700 million, respectively [1]. Obesity is one of the main causes of metabolic diseases including T2DM, steatohepatitis, fatty liver diseases, and many cardiovascular diseases [2, 3]. The reason for this correlation is that both obesity and T2DM are chronic low-grade inflammatory diseases [4]. It is worth noting that macrophages play an essential role in obesity and T2DM. Firstly, the number of macrophages is significantly increased in the adipose tissue of both obese rodents and humans [5]. Further studies showed that the increase in the macrophages is mainly because of the increase of pro-inflammatory macrophages, i.e., the M1-polarized macrophages. The ratio of M1 to M2 macrophages is also increased, which leads to metabolic inflammations in adipose tissues[6]. Chronic tissue inflammation leads to increased levels of pro-inflammatory cytokines, such as TNF-α and IL-1β, which impair insulin signalling and induce insulin resistance [7].

In addition to the classic pro-inflammatory cytokines mentioned above, Pingping Li et al. found that the inflammatory mediator galectin-3 (gal-3), which is mainly secreted by M1-like macrophages in visceral adipose tissues, can inhibit the downstream signalling of the insulin receptor (IR) by directly binding with IR, leading to systemic insulin resistance [8]. These pieces of evidence indicate that modulation of the conversion of M1 to M2-like polarized state of macrophages, either by genetic or pharmacological methods, is a promising approach for the treatment of obesity-induced insulin resistance and diabetes. In this study, the experimental models were obtained by sucrose intoxication. The models show changes have been observed in insulin signalling [9].
Piperine is the major alkaloid present in black pepper (Piper nigrum), long pepper (Piper longum), and many other piper species. Piperine exhibits a wide range of biological properties, such as immunomodulation, anti-oxidation, anti-lipid metabolism disorder, and anti-inflammation [10, 11]. Among these pharmacological activities, what attracts us the most is its excellent modulatory effect on immune inflammation in disease models such as clone diseases, arthritis and ulcerative colitis [12, 13]. Although some previous studies have confirmed the beneficial effect of piperine in improving dyslipidemia and reducing body fat accumulation in sucrose-induced mice[14], it is unclear whether piperine can improve metabolic disorders by inhibiting metabolic inflammation in obesity. Therefore, this study aimed to explore the role of piperine and its implications in the regulation of inflammatory signalling molecules in adipose tissue: A high-fat diet sucrose-induced type-2 diabetic experimental model.

**Materials and Methods**

**Chemicals**

All chemicals and reagents used in this study were purchased from Sigma Chemical Company St. Louis, MO, USA; Invitrogen, USA; Eurofins Genomics India Pvt Ltd, Bangalore, India; New England Biolabs (NEB), USA; Promega, USA. piperine was procured from Sigma Chemical Company St. Louis, MO, USA; Total RNA isolation reagent (TRIR) was purchased from Invitrogen, USA. The reverse-transcriptase enzyme (MMuLv) was purchased from Genet Bio, South Korea purchased from Promega, USA.

**Animals**

The present experimental study was approved by the institutional animal ethics committee (IAEC no.: BRULAC/SDCH/SIMATS/IAEC/07-2019/028). Adult male Wistar albino rats, weighing 180–200g, were obtained and maintained in clean propylene cages at the Biomedical Research Unit and Laboratory Animal Centre (BRULAC), Saveetha Dental College and Hospitals, Saveetha University, India) in an air-conditioned animal house, fed with standard rat pelleted diet (Lipton India Ltd., Mumbai, India), and clean drinking water was made available ad libitum. Rats were divided into 3 groups, each consisting of 6 animals.

**Experimental Design**

The animals were divided into four groups each consisting of six animals:

- **Group-I**: Normal control rats fed with normal diet and drinking water;
- **Group-II**: Type-2 diabetic rats induced by high-fat diet
- **Group-III**: Diabetic (type-2) rats treated with piperine (40 g/kg body weight/day), orally for 30 days; and
- **Group-IV**: Diabetic (type-2) rats treated with metformin (50 mg/kg body weight/day), orally for 30 days.

At the end of the treatment, animals were anaesthetized with sodium thiopentone (40 mg/kg b.wt), blood was collected through the cardiac puncture, sera were separated and stored at –80°C and 20 ml of isotonic sodium chloride solution was perfused through the left ventricle to clear blood from the organs. Liver tissues from control and experimental animals was immediately dissected out and used for assessing the various parameters.

**Assessment of Fasting Blood Glucose (FBG)**

After the overnight fasting, the blood glucose was estimated using On-Call Plus blood glucose test strips (ACON Laboratories Inc., USA). From the rat tail tip, the blood was collected and results were expressed as mg/dl.

**Oral Glucose Tolerance Test (OGTT)**

For the oral glucose tolerance test, animals were fasted overnight. After giving the oral glucose load (10 ml/kg; 50% w/v), blood glucose level was estimated at various time points.
periods (60, 120, and 180 min) by using On-Call Plus blood glucose test strips. Before giving glucose load, the value of blood glucose is considered as 0 min value. Results were marked as mg/dl.

**Isolation of Total RNA**

Total RNA was isolated from control and experimental samples using a TRIR (total RNA isolation reagent) kit. Briefly, 100 mg fresh tissue was homogenized with 1 ml TRIR and the homogenate was transferred immediately to a microfuge tube and kept at -80°C for 60 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, vortexed for 1 min and placed on ice at 4°C for 5 min. The homogenates were centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 sec and placed on ice at 4°C for 10 min. The samples were centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 x g (4°C). The supernatant was removed and RNA pellets were mixed with 50 μl of autoclaved Milli-Q water and dissolved by heating in a water bath for 10 min at 60°C.

**Quantification of RNA**

The diluted RNA sample was quantified spectrophotometrically by measuring the absorbance (A) at 260/280 nm. 40 μg of RNA in 1 ml gives one absorbance at 260 nm. Therefore, the concentration of RNA in the given sample can be determined by multiplying its A260 by 40 and dilution factor. The purity of RNA preparation can be calculated using the ratio between its absorbance at 260 and 280 nm. A ratio of absorbance at 260/280 nm > 1.8 is generally considered good-quality RNA (Fourney et al., 1988). The purity of RNA obtained was 1.8.

**Reverse Transcriptase – Polymerase Chain Reaction (RT – PCR)**

RT-PCR is an approach for converting and amplifying a single-stranded RNA template to yield abundant double-stranded DNA products. 1. First strand reaction: Complementary DNA (cDNA) is made from the mRNA template using Oligo dT, dNTPs & reverse transcriptase. 2. Second strand reaction: After the reverse transcriptase reaction is complete, standard PCR (called the “second strand reaction”) is initiated. Principle RT-PCR is a method used to amplify cDNA copies of RNA. It is the enzymatic conversion of mRNA into a single cDNA template. A specific oligodeoxynucleotide primer is hybridized to the mRNA and is then extended by an RNA-dependent DNA polymerase to create a cDNA copy. First-strand DNA synthesis The RT kit was purchased from Eurogentec (Seraing, Belgium). Reagents 1. 10X RT buffer: One vial containing 1.4 ml of 10X RT buffer. 2. EuroScript reverse transcriptase: One tube containing 75 μl of Moloney Murine leukemia virus reverse transcriptase (3750 U at 50 U/μl).

**Quantitative Real-Time PCR Principle**

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. There are three major steps in a PCR, which are as follows: Denaturation at 94°C for 3 min: During the denaturation at 94°C for 2-5 min, the double strand melts open to single-stranded DNA, and all enzymatic reactions stop. Annealing at 54°C- 65°C for 30 sec: Ionic bonds are constantly formed and broken between the primer and the single-stranded template to ensure the extension process. Extension at 72°C for 30 sec: Primers that are in positions with no exact match get loose again (because of the higher temperature) and don’t give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3’ side (the polymerase adds dNTP from 5’ to 3’, reading the template from 3’ to 5’ side; bases are added complementary to
the template). Because both strands are copied during PCR, there is an exponential increase in the number of copies of the gene.

**Reagents**

1. 2X Reaction buffer: The PCR master mix kit was purchased from Takara Bio Inc., Japan. Contains TaKaRa Ex Taq HS (a hot start PCR enzyme) dNTP Mixture, Mg2+, Tli RNase H (a heat-resistant RNase H that minimizes PCR inhibition by residual mRNA), and SYBR Green I.
2. Forward primer (10µM)
3. Reverse primer (10µM)
4. cDNA- Template
5. Autoclaved milli Q water
6. Primers: The following gene specific oligonucleotide primers were used.

**Procedure**

Procedure Real-Time PCR was carried out on CFX 96 Real-Time system (Bio-Rad). The reaction mix (10 µl) was prepared by adding 5 µl of 2X reaction buffer, 0.1 µl of sense and antisense primer, 1 µl of cDNA and 3.8 µl of sterile water. The thermal cycler protocol was as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles of PCR, denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 20 sec. All reactions were performed in triplicate along with no template control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 50-95°C for each sample to determine the presence of multiple amplicons, non-specific products and contaminants. The results were analysed using CFX 96 Real Time system software (Bio-Rad). As an invariant control, the present study used rat β-actin.

**Results and Discussion**

We tested the action of piperine in the regulation of inflammatory signalling molecules in adipose tissues. Effect of piperine on mRNA expression of TNF alpha in type-2 diabetic rats. revealed that Piperine showed significant control compared to the standard metformin. The result was Significant with P <0.05 (Figure 1). Similarly, the Effect of piperine on mRNA expression of NF kB in type-2 diabetic rats also revealed that Piperine showed significant control compared to the standard metformin. The result was Significant with P <0.05 (Figure 2).

![Figure 1. Effect of Piperine on mRNA Expression of TNF Alpha in Type-2 Diabetic Rats](image-url)
Results obtained in our study were similar to the results revealed in a study by Choi et al., which revealed that the supplement of piperine in a high-fat diet significantly reverses hepatic steatosis and insulin resistance in HFD obese mice[15]. Results obtained in another study by Agarwal H et al in 2017 also showed piperine treatment could lead to moderate body weight loss, significantly reverse glycolipid metabolism disorders, and improve the established insulin resistance and glucose intolerance in MSG-obese mice. Another study demonstrated that Piperine served as an immunomodulator for the treatment of obesity-related diabetes through its anti-inflammatory effects, which might be achieved by inhibiting macrophage M1 polarization in adipose tissues [16].

The strength of this study is the fact that it is based on natural alternatives for synthetic components, which is the new fad among researchers in the field of biomedical applications and technology. The benefits of green medicine are numerous [17]. Hence, with low cost and more benefits, this study can be useful in the field of biotechnology for the upcoming generation. Further, the natural method is convenient, eco-friendly and can be applied in various applications and the use of Piperine Vera has added advantages as the plants have many useful medicinal properties. Building upon our earlier discovery on anti-inflammatory components [18, 19], we anticipate that this plants based anti-inflammatory approach will empower an exciting new area of investigation to design and develop immunotherapeutics for treating human inflammatory diseases.

However, limitation of the study is the fact that it was conducted in vitro, so it cannot be assumed that the results of Anti-inflammatory effects, as well as the other results, could be translated into clinical effectiveness. Recommendations put forward from this study in the field of dentistry is this product can be given to the patients in the required conditions, however along with further in vivo studies with people’s acceptance values as well [20]. It can also be used as an alternative to commercially available products in biomedicine.

Conclusion

For the first time, we have demonstrated that piperine has anti-inflammatory effects. Our results suggest that piperine has the potential as a therapeutic drug or dietary supplement. Thus, further investigations should focus on the development of piperine analogues that have potent efficacy and fewer adverse effects.

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Conflict of Interests

None declared.
References


