EFFECT OF SUGNIL, A TRADITIONAL ANTIDIABETIC HERBAL FORMULATION ON THE EXPRESSION OF TRANSCRIPTION FACTORS IN STREPTOZOTOCIN DIABETIC RATS.

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ABSTRACT

Objective: A number of large randomized clinical trials demonstrated that lipid lowering successfully reduced cardiovascular events and death in patients with diabetes. Recent studies have reported that multiple transcription factors are involved in regulation of lipid metabolism in diabetic subjects. Numerous plant extracts and formulations have been shown to regulate the expression of transcription factors which in turn normalize lipid abnormalities in diabetic patients. In this series, sugnil, a traditional antidiabetic polyherbal formulation was assessed for its regulatory effect on the expression of transcription factors in lipid metabolism of streptozotocin induced diabetic rats.

Methods: The mRNA expressions of fatty acid synthesis related genes such as SREBP-1 and SREBP-2 and fatty acid decomposition related gene PPAR-α were measured by real-time RT-PCR using an appropriate primers and thermal cycler conditions.

Results: Oral administration of sugnil to diabetic rats for 42 consecutive days effectively normalized the expression of these transcription factors to near normal as in control rats. Expression of PPAR-α was found to be up-regulated that leads to diminished expression of SREBP-2 in the liver of diabetic rats treated with sugnil. Treatment with sugnil also down regulated the SREBP-1 expression in the kidney of diabetic rats.

Conclusion: The molecular mechanisms of action of sugnil in the regulation the lipid profile in diabetic rats might be normalizing the expression of these transcription factors.

Keywords: Transcription factors, PPAR-α, SREBP-1, SREBP-2.

INTRODUCTION

Lipid abnormalities in diabetes mellitus results in increased risk of cardiovascular diseases, coronary insufficiency, myocardial infarction. Cardiovascular complications especially atherosclerosis has become a predominant cause of death in diabetic patients. Lipid management has been considered as an effective approach to reduce cardiovascular risk in diabetes subjects [1]. Thus, to reduce the risk of late complications and negative outcome of diabetes mellitus, not only the control of blood glucose levels, but also lipid levels is necessary. A traditional antidiabetic polyherbal formulation, marketed in the brand name of sugnil is selected for current investigation. Sugnil consists of ingredients from nine medicinal plants viz Aristolochia bracteata, Balsamodendron mukul, Casearia esculenta, Cassia auriculata, Coscinium fenestratum, Curcuma longa, Eugenia jambolana, Gymnema sylvestre, and Triphala. Preparation of Sugnil is based on traditional methods in accordance with the procedure suggested in the antique literature (Table 1). Our previous study has reported the antihyperlipidemic potency of sugnil in streptozotocin induced diabetic rats [2]. In this study, an attempt has been made to investigate the molecular mechanism of sugnil action in lowering lipid levels in experimental animals.

Changes in gene expression are an important component of the pathogenesis of diabetes [3,4]. Multiple transcription factors are involved in the regulation of both carbohydrate and lipid metabolism either directly or through interaction with insulin and a large number of insulin-regulated genes have been identified in liver and adipose tissue [5]. Transcription factors are soluble proteins that are able to bind to DNA. Their binding to promoter sites of genes influences the transcription of genes, leading to up- or down-regulation of gene expression. In recent years, numerous plant extracts and plant formulations have been shown to regulate the expression of transcription factors and genes in metabolic pathways of different diabetic animal models [6-9]. In the present study, the effect of sugnil on the expression of transcription factors such as peroxisome proliferator-activated receptor-α (PPAR-α), sterol regulatory element-binding protein-2 (SREBP 2) and sterol regulatory element-binding protein-1 (SREBP 1) were assessed in streptozotocin induced diabetic rats using RT-PCR assay.

Peroxisome proliferator-activated receptor (PPAR) belong to a superfamily of nuclear, ligand-activated transcription factors, which play an important role in the transcriptional regulation of genes responsible for the control of lipid utilization and storage, and modulation of lipoprotein metabolism, adipocyte differentiation and insulin action [9]. PPARs consist of three nuclear receptor isoforms, PPAR alpha, PPAR delta and PPAR gamma, encoded by separate genes [10]. PPAR-α isoform is predominantly expressed in the liver and regulates the transcription of genes involved in hepatic fatty acid uptake and oxidation [11]. PPAR-α agonists have been used for over 40 years in the treatment of dyslipidemia, mainly due to their hypolipidemic actions, such as lowering triglyceride (TG) and raising high density lipoprotein levels [12]. However, these agents also produce hepatic peroxisome proliferation, hypertrophy and hyperplasia in rodents [13]. Alternatively, numerous plant extracts and traditional plant formulations have been reported to have PPAR-α modulatory activity in different diabetic animal models [14].

Sterol regulatory element-binding proteins (SREBP)s are transcription factors that regulate fatty acid and cholesterol synthesis. SREBP are belong to basic helix-loop-helix-leucine zipper family and activate the entire program of fatty acid and cholesterol synthesis in liver [15,16]. Three isoforms of SREBP are known in mammals, SREBP-1a, SREBP-1c and SREBP-2. In most animal tissues, SREBP-1c is the predominant SREBP-1 isoform [17]. Multiple lines of evidence suggest that SREBP-1 and SREBP-2 have different relative effects on target genes. SREBP-1 preferentially activates genes involved in fatty acid synthesis, including acetyl CoA carboxylase and fatty acid synthase (FAS), whereas SREBP-2 preferentially activates genes involved in cholesterol biosynthesis such as hydroxymethylglutaryl CoA synthase, hydroxymethylglutaryl CoA reductase, farnesyl diphosphate synthase, and squalene synthase [18].
Animal groups for experimental studies

Rats were acclimatized to the laboratory conditions at least for a week and they were divided into four groups of with four rats in each group as follows,

**Group I:** Control rats administered 0.5 ml of saline for 42 days.

**Group II:** STZ-induced diabetic control rats administered 0.5 ml of saline for 42 days.

**Group III:** STZ-induced diabetic rats administered sugnil (100 mg/kg body weight) for 42 days.

**Group IV:** Control rats administered sugnil (100 mg/kg body weight) for 42 days.

Analysis of mRNA expressions

The STZ-induced diabetic rats were administered with sugnil for 42 consecutive days. At the end of treatment schedule, two rats from four different experimental groups were sacrificed under mild anesthesia (non-fast condition), the liver and kidney tissues were immediately removed, instantly soaked in liquid nitrogen and stored at -80°C for study of mRNA using RT-PCR.

**1 - RNA Extraction from tissue samples**

Total mRNA was prepared separately from the tissues of individual rats in different experimental groups using TRIzol reagent.

**Homogenization**

About 15 mg of frozen liver tissue and 75 mg of frozen kidney tissue were homogenized separately in 1 ml of TRIzol reagent using a glass–Teflon homogenizer.

**Phase separation**

The homogenized tissue samples were incubated for 5 minutes at room temperature (30°C) for complete dissociation of nucleoprotein complexes. After incubation, 0.2 ml of chloroform was added and the tubes were shaken vigorously by hand for 15 seconds. The entire content was then centrifuged at 12,000 x g for 10 minutes at 8°C. Following centrifugation, the mixture was separated into a lower red, phenol–chloroform phase, an interphase and a colorless upper aqueous phase. RNA was present exclusively in an aqueous phase and was transferred to a fresh tube.

**RNA precipitation**

RNA from the aqueous phase was precipitated by mixing with 0.5 ml isopropanol alcohol. The samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 8°C. After centrifugation RNA precipitate was formed as gel like pellet on the side and bottom of the tube.

**RNA wash**

The pellet was collected and 1 ml of 75% ethanol was added. The samples were vortexed and then centrifuged at 7,500 x g for 5 minutes at 8°C.

**Redissolving the RNA**

At the end of the procedure, RNA pellet was air dried, and dissolved in RNase – free water. The concentration and purity of RNA preparation were checked by measuring the absorbance at 260 nm and 280 nm.

The above mentioned RNA extraction procedure was performed individually to all the tissue samples dissected from rats in four different experimental groups. The extracted RNA’s were used as template for the synthesis of first-strand of cDNA.

**II - First Strand cDNA Synthesis**

cDNA Synthesis was performed using superscript III first-strand synthesis system for RT-PCR (Invitrogen, USA) following manufacturer’s protocol as follows;

As a first step, the following components were added into a thin-walled 0.5 ml PCR tube to prepare cDNA synthesis mix. 1µg total RNA (extracted separately from tissues of rats in four different
Fatty acid synthesis related genes – SREBP-1 and SREBP-2

The sequences of these genes were used to design primer for the RT-PCR. Primer Design was used as a template for RT-PCR. The Primer sequences for the previous work by scientists and from Gene bank database (http://www.ncbi.nlm.nih.gov/).

Fatty acid synthesis related genes – SREBP-1 and SREBP-2

Fatty acid decomposition related gene – PPARα

The housekeeping gene, β Actin

The sequences of these genes were used to design primer for the RT-PCR. The primers were ordered from GeNei, Bangalore. 1 µl of cDNA was used as a template for RT-PCR. The Primer sequences for the above mentioned genes are as follows;

<table>
<thead>
<tr>
<th>Genes</th>
<th>Denaturation temperature</th>
<th>Annealing temperature</th>
<th>Extension temperature</th>
<th>No. of cycles</th>
<th>Final extension</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>SREBP-1</td>
<td>95°C for 15s</td>
<td>64°C for 40s</td>
<td>72°C for 30s</td>
<td>30</td>
<td>72°C for 10m</td>
<td>72Bp</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>94°C for 1m</td>
<td>65°C for 1m</td>
<td>72°C for 1m</td>
<td>30</td>
<td>72°C for 10m</td>
<td>416Bp</td>
</tr>
<tr>
<td>PPARα</td>
<td>94°C for 30s</td>
<td>64°C for 30s</td>
<td>72°C for 1m</td>
<td>35</td>
<td>72°C for 10m</td>
<td>425Bp</td>
</tr>
<tr>
<td>β ACTIN</td>
<td>94°C for 30s</td>
<td>60°C for 30s</td>
<td>72°C for 1m</td>
<td>35</td>
<td>72°C for 10m</td>
<td>357Bp</td>
</tr>
</tbody>
</table>

Table 2: Thermal cycler conditions for selected genes

V - Gel Electrophoresis and data acquisition

The PCR amplified products of the selected genes were electrophoresed using 1.0% agarose gel prepared in 1X Tris acetic acid EDTA buffer [0.5g of agarose dissolved by heating in 50 ml of 1X TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0)]. A dye solution consisting of bromophenol blue (0.25%), Xylene cyanol FF (0.25%) and 40% sucrose in water was added at 3 µl per DNA sample prior to electrophoresis. Electrophoresis was carried out in a horizontal electrophoresis unit. The agarose gel was formed using a gel caster and the dimensions of the gel were adjusted to 9.6 by 14.2 by 0.6 cm. The wells were made by using comb, the ladder DNA (10 µl) and samples (10 µl each) were loaded at wells using micropipette in the following manner.

Lane 1 – DNA ladder sequence (1000 bp).

Lane 2 – Amplicon of selected gene from control rats.

Lane 3 – Amplicon of selected gene from diabetic control rats.

Lane 4 – Amplicon of selected gene from control rats treated with sugnil (100 mg/kg).

Lane 5 – Amplicon of selected gene from control rats treated with sugnil (100 mg/kg).

The gel was allowed to run at 85 V, maximum Amp and maximum Watts for 1 hour 20 minutes or till the dye neared at the bottom of the gel using the high voltage power supply unit. The gel was then placed in the ethidium bromide staining solution (0.01 g/L) for 20 minutes and transferred to destaining solution (1X TAE) for 30 minutes. The resolved DNA in the gel was visualized under UV transilluminator. Electrophoresis was carried out individually for all the selected genes to separate their PCR amplicons. At the end, the gels were photographed using a gel documentation system (Bio Rad, CA, USA) and the density of the PCR products was measured using a GS-700 imaging densitometer. The housekeeping gene, β actin was amplified as a control gene (Fig. 1b, 2b & 3b). The level of mRNA was expressed as the ratio of signal intensity for each gene relative to that of β actin. All results were confirmed with at least three independent experiments.

RESULTS

Expression of mRNA in Liver

In the liver, expressions of fatty acid decomposition related gene PPARα and fatty acid synthesis-related gene SREBP-2 were measured by real-time RT-PCR. The mRNA expression of PPARα was found to be decreased, whereas the expression of SREBP-2 was significantly increased in streptozotocin induced diabetic rats when compared to normal control rats. Oral administration of sugnil for 42 consecutive days effectively reversed the expression pattern of these genes to near normal as in the control rats. However, no significant difference was found in the expression pattern of these genes in control rats treated with sugnil (Fig. 1a & 1c and 2a & 2c).

Expression of mRNA in kidney

In the kidney, the mRNA expression of SREBP-1 was significantly increased in streptozotocin induced diabetic rats when compared to normal control rats. Oral administration of sugnil for 42 consecutive
days significantly decreased the expression of this gene to near normal as in control rats. However, no significant difference was found in the renal expression of SREBP-1 in control rats treated with sugnil (Fig 3a&3c).

**DISCUSSION**

To investigate the lipid lowering effects of sugnil changes in the expression of transcriptional factors involved in hepatic lipogenesis were examined using RT-PCR assay. Peroxisome proliferator-
activated receptor (PPAR), a ligand activated transcription factor, is highly expressed in liver, heart, and skeletal muscle [20]. PPAR-α-mediated responses have been traditionally studied in the liver. PPAR-α activation mediates expression of genes regulating lipid oxidation [21]. Both, natural and synthetic ligands are found to activate PPAR-α and thereby regulate the lipid metabolism in man and animals [6,14]. In recent years, many plant extracts and traditional plant formulations have been shown to possess PPAR-α modulatory activity in different diabetic models [14].

In this context, sugnil, a traditional anti diabetic polyherbal formulation was examined for its PPAR-α modulatory effect in streptozotocin induced diabetic rats. Results of this study showed that the expression of PPAR-α was decreased in streptozotocin induced diabetic rats. This effect may be due to the hyperglycemic condition caused by streptozotocin and/or loss of insulin signaling. However, oral administration of sugnil for 42 consecutive days effectively increased the expression of PPAR-α in the liver of STZ-induced diabetic rats. This possible effect of sugnil may be due to the presence of bioactive compound(s), which could act as a ligand for PPAR-α activation. The activated transcription factor PPAR-α induces the mRNA expressions of target genes responsible for fatty acid β oxidation. This was evidenced by normal levels of triglycerides and high density lipoproteins observed in sugnil treated diabetic rats [2]. Further, it has been reported that PPAR-α activation lowers the cholesterol concentration in rat liver, plasma and lipoproteins by reducing the amount of nuclear SREBP-2 in the liver of STZ-induced diabetic rats [2]. Consistent with this finding, the present study results also showed that the activated PPAR-α decreased the expression of SREBP-2 in the liver of sugnil treated diabetic rats. This might be a reason beyond the decreased concentrations of total cholesterol observed in the liver and plasma of sugnil treated diabetic rats [2].

Over all, these results strongly suggest that sugnil behave like natural ligand for PPAR-α activation. This leads in turn to the diminished expression of SREBP-2. Hence, the molecular mechanism of sugnil action in the regulation of circulatory and hepatic lipid metabolism in diabetic rats might be modulation of PPAR-α and SREBP-2 gene expression.

SREBP-1 plays an important role in the regulation of lipid homeostasis in kidney; an altered regulation of this transcription factor may be involved in diabetic nephropathy by modulating renal lipid metabolism. A previous study has shown that the expression of SREBP-1 was increased in the kidney cortex of STZ-induced diabetic rats, resulting in up-regulation of enzymes responsible for fatty acid synthesis and, as a consequence, high renal triglyceride content, and was associated with mesangial expansion and glomerulosclerosis. Treatment of diabetic rats with insulin to correct the hyperglycemia prevented the increase in renal expression of SREBP-1 and the renal accumulation of triglyceride [23]. The result of the present study showed that the expression of SREBP-1 was found to be increased in the kidney cortex of STZ-induced diabetic rats. Hyperglycemic condition caused by streptozotocin might be responsible for this effect. However, oral administration of sugnil for 42 consecutive days effectively decreased the expression of SREBP-1 in the kidney of STZ-induced diabetic rats. This possible effect of sugnil might be due to the stimulation of insulin secretion from pancreatic β cells, since insulin was reported to normalize SREBP-1 expression in STZ-induced diabetic rats [23]. Insulin stimulatory effect was directly witnessed by increased concentration of serum insulin reported in sugnil treated diabetic rats [24]. Therefore, the results suggest that down-regulation of renal SREBP-1 through insulin stimulation might be the mechanism of sugnil action in lowering renal lipid contents.

CONCLUSION

The regulation of lipid metabolism by sugnil might involve up-regulation of PPARα and diminished expression of SREBP-2 in liver and SREBP-1 in kidney of diabetic rats. The bioactive compound(s) present in sugnil might act as a natural ligand and normalized the expression of these transcription factors. These possible effects of sugnil may arrest the development and progression of atherosclerosis and related cardiovascular complications in diabetes mellitus. Hence, its therapeutic usage can improve the quality of life in diabetic patients.

CONFLICT OF INTERESTS

 Declared None

REFERENCES


