Dietary coconut kernel protein beneficially modulates NFκB and RAGE expression in streptozotocin induced diabetes in rats

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Abstract Previous studies showed that arginine rich coconut kernel protein (CKP) maintains glucose homeostasis in experimental diabetic rats. But the mechanism of this effect was not clear. This study investigated the effect of CKP on the expression of liver receptor for advance glycosylated end products (RAGE), inducible nitric oxide synthase (iNOS) and NFκB. Diabetes was induced by injecting a single dose of streptozotocin (75 mg/kg body weight) intraperitoneally. After inducing diabetes, CKP was administered to rats orally for 45 days. After the experimental period, serum glucose, insulin, liver glycogen, glucose metabolizing enzyme activities and the expression of liver RAGE, iNOS and NFκB was evaluated. The results showed that CKP beneficially modulated the levels of glucose and insulin as well as the metabolizing enzyme activities. Expression of RAGE and NFκB was found to be over expressed in diabetic rats but was found to be down regulated in CKP fed diabetic rats. iNOS expression was down regulated in diabetic rats, which was expressed normally in CKP fed diabetic rats.

These results clearly demonstrated that anti diabetic activity of CKP is mediated through NFκB pathway.

Keywords Coconut protein · iNOS · RAGE · NFκB · Diabetes · Streptozotocin

Introduction

Diabetes is characterized by hyperglycaemia resulting in various short-term metabolic changes in lipid, protein metabolism and long-term irreversible vascular changes (Barry et al. 2002). Pharmacological studies have proved that many peptides isolated from plants have potential biological effects (Liang et al. 2006; Tepkeeva et al. 2009; Xudong et al. 2006). These peptides have a number of advantages over other chemical agents including their low molecular weight, relatively simple structure, lower antigenicity and fewer adverse actions. However, studies of the peptides from plants have not progressed as quickly as in the case of other compounds. Therefore, it is worthwhile emphasizing the importance and the potential future of research involving biologically active peptides from plant sources. Coconut fruit is composed of about 38.5 % shell, 51.7 % kernel and 9.8 % water (Vigila and Baskaran 2008). The kernel contains 22.8 % crude protein which is very low compared to other vegetable protein sources (Moorthy and Viswanathan 2009). Among the amino acids present, arginine and glutamic acid was found to be the predominant in coconut kernel protein (CKP) (Salil et al. 2011). Previous experiments showed that CKP isolated from coconut kernel had beneficial anti peroxidative and cardioprotective effect on alcohol and isoproterenol treated rats as well as on human volunteers (Nair et al. 1998a, b; Mini and Rajamohan 2004, 2002).

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Both clinical and experimental studies have shown beneficial effects of L-arginine administration in improving vascular function in diabetic conditions (West et al. 2008; Giugliano et al. 1997; Tanju O' zc, elikay et al. 1999). However, the mechanism for the action of arginine in enhancing endothelial NO synthesis remains unknown (Kohli et al. 2004). In view these facts, we designed the study to examine the effect of CKP on the serum glucose and insulin levels as well as the expression of inducible nitric oxide synthase (iNOS), receptor for advanced glycation end products (RAGE) and nuclear factor-kB (NFkB) in streptozotocin induced diabetes in rats.

Materials and methods

Isolation and purification of CKP Coconut kernel (West Coast Tall variety, from the Kerala University campus) was separated, pulverized and defatted using petroleum ether (60–80 °C) for 2–3 h. Defatted material was stirred with 10 % NaCl solution in large containers using mechanical stirrer for 24 h. The saline extract was collected and by adjusting the pH with dilute HCl solution, the proteins were precipitated. The precipitated globulin fraction was collected by centrifugation and redissolved in minimum volume of 10 % NaCl solution. After centrifugation the protein was precipitated and the globulin fraction was collected, washed with distilled water and dried in a vacuum oven at 60 °C and was used for feeding purpose (Salil et al. 2011).

Amino acid composition of CKP Amino acid analysis of CKP showed that it contains 28.8 % non-polar amino acids, equal amounts of proline, glycine, alanine, valine, methionine, isoleucine and leucine. The amount of acidic amino acids viz. glutamic acid and aspartic acid were 24.7 %. Among the basic amino acids, arginine level (17.8 %) was found to be significantly higher (Salil et al. 2011). Electrophoretic separation of CKP showed the presence of 3 bands (Salil et al. 2012).

Animals All the animal cares and procedures were according to the guidelines of the Institutional Animal Ethical Committee (IAEC). One-month-old female Sprague–Dawley rats (150–200 g body weight) bred in our department animal house was used for the study. The animals were housed individually in polypropylene cages in a room maintained at 25±10 °C with a 12 h light and 12 h dark cycle.

Induction of diabetes Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of freshly prepared streptozotocin (75 mg/kg body weight). Diabetes was confirmed in the treated rats by measuring the fasting blood glucose concentration 48 h after injection. Rats with

fasting blood glucose of more than 200 mg/dl was considered diabetic and included in the study (Larrieta et al. 2006)

Animal experiments To study the effect of CKP feeding on carbohydrate metabolism and the expression of NFkB and iNOS, the rats were divided into 3 groups of six animals each: **Group I- Normal control**, **Group-II- Diabetic control**, **Group III- Diabetic administered 8 % CKP**. All the animals received a normal laboratory diet. Group III animals received CKP orally. The experiment lasted for a period of 45 days. After the experimental period, animals were fasted overnight and sacrificed by sodium pentothal injection. Blood and tissues were collected for various estimations. All the animal cares and procedures were according to the guidelines of the Institutional Animal Ethical Committee (IAEC).

Serum glucose and insulin levels Estimation of serum glucose was done using a commercial kit based on glucose oxidase method of Lott and Turner (1975). Reagent consists of 92 mM Tris buffer (pH 7.0), 0.3 mM phenol, 15,000 U/L glucose oxidase, 2.6 mM 4-aminoantipyrene. Briefly, 0.1 ml of serum was mixed with the reagents and incubated for 10 min at 37 °C. After 10 min the quinonimine formed was measured at 505 nm. Serum insulin was measured with an automated immunochemiluminometric (ICL) assay provided by Bayer Diagnostics (ADVIA Centaur insulin assay) according to the manufacture's instruction.

Serum fructosamine level Serum Fructosamine was estimated by measuring the reducing activity of nitroblue tetrazolium (NBT) method using the kit from Spinreact, S.A, Spain (Schleicher and Vogt 1990). Briefly, 1 mL of working reagent was taken in tubes marked blank, standard and test. To the test added 100 ml of sample and to the standard added 100 ml of fructosamine calibrator. Mixed well and incubate at 37 °C. The absorbance of the test and the standard was taken at 520 nm exactly after 10 min and 15 min against the blank.

Glycosylated hemoglobin content Haemolysates of erythrocytes were prepared by the method of Trivelli et al. 1971 with slight modifications. Briefly, blood samples were drawn by ocular venipuncture into evacuated tubes containing EDTA. Blood was centrifuged at 800 × g for 10 min at 4 °C to remove plasma and packed cells. Packed cells were lysed with two volumes of water. One volume of carbon tetrachloride was added to the hemolysate, mixed and refrigerated at 4 °C overnight. Lysate was centrifuged at 27000 × g for 30 min at 4 °C, supernatant removed and stored at −70 °C until analysis. Total hemoglobin in the blood sample was determined by the method of Varley et al. 1980. Colorimetric estimation of glycated hemoglobin

was measured according to the procedure of Parker et al. 1981. 1 ml of diluted hemolysate (10 mg of hemoglobin or fructose standard) was mixed with 1 ml of oxalic acid reagent. Mixture was incubated for 60 min at 124 °C in sealed tubes. After incubation, mixture was allowed to cool to room temperature and added 1 ml of the trichloroacetic acid to each tube, mixed and filtered through a 15 cm×0.5 (1.d) glass column with a glass wool plug in the bottom. To 1.5 ml eluate add thiobarbituric acid reagent (0.05 M) and measured the absorbance at 443 nm.

Liver glycogen content Glycogen was isolated from fresh liver tissues by adding 30 % KOH, 0.5 ml of saturated Na₂SO₄, 1 ml of 95 % ethanol and centrifuged at 2000 rpm for 10 min. The resulting pellet was redissolved in saline. From this 0.1 ml aliquot was taken and 2.5 ml anthrone was added, boiled at 90 °C for 10 min, cooled in ice and absorbance was measured at 660 nm (Carroll et al. 1956).

Assay of hexokinase and pyruvate kinase The chilled tissue was homogenized at 0 °C with 3 volumes of buffer of the following composition. Tris 0.1 M, Histidine–0.1 M, EDTA–0.01 M and MgCl₂–0.01 M (pH 7.0). The homogenate was centrifuged at 5000xg at 0 °C for 5 min and the supernatant was used as enzyme source. Hexokinase activity was assayed by the method described by Crane and Sols 1953.²² The reaction mixture contains the following components; 0.2 ml of ATP: MgCl₂ (0.075 M ATP and 0.04 M MgCl₂), 0.2 ml Buffer mixture (solution of Tris, histidine, EDTA, and MgCl₂ of appropriate strength were mixed, neutralized to pH 7.0 and diluted to the appropriate concentrations.), 0.2 ml 0.01 M glucose and 0.3 ml. The tubes were preincubated at 30 °C and the reaction was started by the addition of 0.1 ml enzyme. After 1 min an aliquot was withdrawn to ice cold solution of 5 % ZnSO₄. It was then neutralized with 0.3 N BaOH₂ using phenolphthalein as indicator, centrifuged and the glucose in the supernatant was estimated. Serum pyruvate kinase activity was assayed in a coupled enzymic reaction in 100 mM-Tris/HCl buffer, pH7.4, containing 100 mM-KCl, 4 mM-MgCl₂, 1 mM-fructose 1,6-bisphosphate, 0.15 mM-NADH, 2 mM-ADP and 5 units of lactate dehydrogenase. The assay was initiated by the addition of 1 mM-phosphoenolpyruvate and performed at 270 °C. One unit of pyruvate kinase activity converts 1 μmol of phosphoenolpyruvate into pyruvate/min (Ishibashi and Cottam 1978).

Expression of RAGE, NFκB, AND iNOS Total RNAs was isolated from livers using Axy prep multisource total RNA miniprep kit (Axygen biosciences). RNA isolated was synthesized to cDNA using Fermentas Revert Aid First strand cDNA Synthesis. The PCR amplification was carried for

three different transcripts, RAGE, NFκB and iNOS. The primers employed for amplification was purchased from Biogene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was designed as the house keeping control gene. The sequences of the primers are shown in Table 1. PCR amplification was carried out using Fermentas PCR Master Mix (2X) kit in Master cycler Personal (Eppendorf). The 50 μl reaction mixture contained 25 μl PCR master mix, 1.5 μl of each primer (forward and reverse) 3 μl cDNA template and nuclease free water. The cycle profile was as follows: activation step of polymerase i.e., 3 min at 94 °C followed by 39 cycles of 30 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C. 39 cycles were followed by a hold at 4 °C. The PCR Products were run on 1.5 % agarose gel and exposed to UV in GEL/CHEMI DOC (Biorad laboratories). The intensity of the band quantified using Quantity One 1D analysis software. The quantification of a particular band was expressed as a ratio with the control.

Statistical analyses The results are expressed as the mean values with their standard errors. Intergroup comparison was performed by one-way ANOVA followed by Duncan's variance. Significance was set at $p < 0.05$. Experiments were carried out 3 times.

Results and discussion

Effect on insulin, glycogen and sugar levels Figure 1 Shows the levels glucose in the serum of control and CKP treated diabetic rats. Diabetic control animals showed significant increase in glucose level and sharp decrease in the insulin levels compared to normal control animals (Fig. 1). Diabetic rats fed CKP showed significant decrease in glucose levels with an increase in the serum insulin levels. Levels of liver glycogen was significantly decreased in diabetic control, while it was found to be restored to the basal levels in CKP fed rats (Fig. 1).

Table 1 Sequences of primer pairs used for real time quantitative RT-PCR

Name	Primer pairs
RAGE	Forward: 5'-tcagatcccgaacgcta- 3' Backward: 5'- tgacacaaggcctccaa-3'
NFκB	Forward: 5'-ttgccgtgatcaccaa-3' Backward: 5'- caagagtcgtccagtc-3'
iNOS	Forward: 5' -ctgttcaccaggagatgttg - 3' Backward: 5'-ctcctgccccctgagttcgtc- 3'
GAPDH	Forward: 5'-atcccatcaccattctcaag-3 Backward: 5'-cctgttcaccaccattcttg-3'

Fig. 1 Serum glucose, insulin, serum glycated hemoglobin, fructosamine and liver glycogen levels in control and CKP fed diabetic rats ($n=6$ rats). Values are mean $\pm$ SEM of 6 rats, $p<0.05$, Group I- Normal control, Group II- Diabetic control, Group III- Diabetic + CKP. *Significant compared to Group I, **Significant compared to Group II

Injection of streptozotocin has reduced the activities of plasma hexokinase and pyruvate kinase, while diabetic rats feed CKP has restored the activities of these enzymes to the basal levels. Streptozotocin is a diabetogenic drug that disrupts glucose and energy metabolism in brain and other organs (Muller et al. 1998). Feeding CKP could probably interfere in the pancreatic neogenesis and thereby induce the secretion of insulin and carbohydrate metabolism. Furthermore, CKP may also be utilized in the synthesis of proteins. Due to the high prevalence of diabetes worldwide, extensive research is still being performed to develop new anti-diabetic agents (ADA 2007; Venkateswaran and Pari 2003; Udayakumar et al. 2009; Samane et al. 2006; Luo and Luo 2006). Modulating dietary proteins and the flux of circulating amino acids generated by their consumption and digestion might underlie powerful new approaches to treat various metabolic diseases such as obesity and diabetes (Tremblay et al. 2007; Wu 2009). Previously we have reported the hypoglycemic effect of CKP in alloxan treated rats, but its effect on nitric oxide pathway and RAGE was unknown. So this study was undertaken to elucidate the mechanism of action of CKP in experimental diabetes.

Effect on different biochemical parameters Levels of glycated hemoglobin and fructosamine was found to be significantly increased in diabetic control animals. The levels of these parameters were found to be decreased to the basal levels in diabetic rats fed CKP (Fig. 1). The important enzymes in glucose metabolism, pyruvate kinase and hexokinase was found to be decreased in diabetic control animals, while the activities of these enzymes was brought towards the basal levels by CKP (Fig. 2).

In experimental diabetes, glucose metabolic enzymes are markedly altered. In the present study, diabetic rats showed significant decrease in the activity of pyruvate kinase and hexokinase in the liver. Hexokinase that catalyses the conversion of glucose to glucose-6-phosphate plays a central role in the maintenance of glucose homeostasis. In the liver, hexokinase is an important regulatory enzyme in the oxidation of glucose (O'Doherty et al. 1999). Being an insulin-dependent enzyme, the hepatic hexokinase activity of diabetic rats is almost entirely inhibited or inactivated due to the absence of insulin (Gupta et al. 1999), since insulin increases hepatic glycolysis by increasing the activity and amount of several key enzymes. This impairment results in a

Fig. 2 Hexokinase and pyruvate kinase activity in control and CKP fed diabetic rats and expression of RAGE, NFκB and iNOS in the liver of control and CKP fed diabetic rats ($n=6$ rats). Values are mean $\pm$ SEM of 6 rats, $p<0.05$, Group I- Normal control, Group II- Diabetic

control, Group III- Diabetic + CKP, iNOS (inducible Nitric Oxide Synthase); RAGE (Receptor Advanced Glycated End Products), GAPDH (Glyceraldehyde 3-Phosphate Dehydrogenase). *Significant compared to Group I, **Significant compared to Group II

marked reduction in the rate of glucose oxidation *via* glycolysis, which ultimately leads to hyperglycemia. Oral administration of CKP to STZ-induced diabetic rats resulted in a significant reversal in the activity of hexokinase, thereby increased the oxidation of glucose.

Expression of NFκB, RAGE and iNOS Expression studies showed that NFκB and RAGE in the liver of diabetic rats were found to be significantly elevated, while the expression of iNOS was significantly decreased in the liver of diabetic control animals compared to normal animals. In CKP fed diabetic animals, NFκB and RAGE expression was significantly down regulated. The expression of iNOS was up regulated in the liver of diabetic rats fed CKP (Fig. 2).

AGE and RAGE were found to co-accumulate with diabetes in renal glomerulus, retina and aorta, and both AGE levels and RAGE expression was reduced by treatment with aminoguanidine (Soulis et al. 1997). The receptor for AGE (RAGE), an integral membrane protein, forming the central binding site on the cell surface for AGEs, is a multi ligand and signal transduction receptor belonging to immunoglobulin superfamily and can be unregulated under various pathological conditions (Katz et al. 2005; Ziyadeh et al. 1997; Park et al. 2004; Schmidt et al. 2000). The engagement of AGEs to RAGE leads to the activation of the transcription factor, nuclear factor kappa B (NFκB) and subsequent

expression of NFκB regulated cytokines. Our results show that untreated diabetic rats have higher glycated Hb and upregulated RAGE and NFκB, while CKP treatment showed a significant down regulation in the levels of NFκB and RAGE in diabetic rats. It is suggested that CKP as a whole or individual amino acids may be responsible for this effect.

The role of NO in the regulation of hemodynamics under hyperglycemic conditions has been controversial. It was found that, apart from endothelial cells, NO from sources such as inducible NOS (iNOS) and neuronal NOS (nNOS) may contribute to increased NO in diabetes (Nagareddy et al. 2005). Increased expression and activity of iNOS in superior mesenteric arteries (SMA) from 12–14 week STZ-induced diabetic rats were reported earlier (Bardell and MacLeod 2001). In our experiments we have found that the levels of iNOS in liver of diabetic rats were significantly lowered than that of normal rats, while CKP feeding restored it towards the normal levels. L-arginine is proved to induce intensive formation of new β cells in diabetic pancreas occurred in a regulated manner since it was accompanied by apoptosis (Vasiljevi et al. 2007). This is in accordance to our previous study, indicating the pancreatic stability in rats fed with L-arginine and CKP, compared to diabetic control. Since there is a modulation in the levels of iNOS and subsequent down regulation of

NFkB, RAGE and glucose metabolism in CKP treated diabetic rats, we speculate that higher amount of arginine may be responsible for the antidiabetic activity of CKP mediated possibly through NFkB pathway.

Conclusion

CKP with its high amount of arginine content, when fed to diabetic rats were found to down regulate NFkB and RAGE and thereby maintain the glucose homeostasis to normal. These results should be extrapolated to human studies so as to develop CKP as a potent functional food against diabetes.

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Conflict of interest statement Authors declare that there is no conflict of interest.

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