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Protective role of tetrahydrocurcumin and chlorogenic acid on glycoprotein changes in streptozotocin-nicotinamide-induced diabetic rats

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Abstract

The present study was to evaluate the individual and synergistic effect of two-plant derived phenolic compounds, namely tetrahydrocurcumin (THC) and chlorogenic acid (CGA) on glycoprotein levels in plasma, liver and kidney of normal and streptozotocin (STZ)-nicotinamide (NA)-induced diabetic rats. A single intraperitoneal injection of STZ (45 mg/kg b.w) to rats, lead to a significant ($p < 0.05$) increase in the levels of fasting plasma glucose and glycoproteins (hexose, hexosamine, fucose and sialic acid), whereas the levels of plasma insulin decreased significantly ($p<0.05$). THC (80 mg/kg b.w) and CGA (5 mg/kg b.w) as individual and combined dose were administered to diabetic rats for a period of 45 days. The levels of plasma insulin, glucose and glycoproteins were measured. From these observed findings, we suggest that the combined THC/CGA treatment shows more effective than individual treatments in decreasing plasma glucose, glycoproteins and increase insulin levels. This shows significant protection of THC/CGA over glycation of membrane proteins against STZ-NA-induced diabetic rats.

Keywords: Chlorogenic acid, glycoproteins, insulin, streptozotocin, tetrahydrocurcumin.

Introduction

Diabetes mellitus is characterized by deranged metabolism of glucose, fats, and proteins [1]. Recent estimations revealed that around 200 million people suffer from diabetes mellitus, making as the most common serious metabolic disorder worldwide [2]. Furthermore, impaired metabolism of glycoproteins plays a major role in the pathogenesis of diabetes mellitus [3]. Under diabetic conditions, reactive oxygen species are produced mainly through glycation reaction [4], which occurs in various tissues [5] and may play an important role in the development of diabetic complications [6]. Advanced glycation end products (AGEs) modify galactose, fucose and sialic acid contents of specific cellular glycoproteins [7].

Glycoproteins are a group of complex proteins containing covalently bound oligosaccharides attached to their polypeptide backbone. Hexose, fucose, hexosamines and sialic acid form the monosaccharide units of oligosaccharide. Glycoproteins are important components of intracellular matrix, cell membrane and membranes of the subcellular organelles. They play a vital role in the maintenance of structural integrity of the membrane bilayer. Cell surface glycoproteins have important roles in the transport of vitamins and lipids, in signal transduction as hormone receptors and in immunological specificity.

Streptozotocin (STZ) (N- (methyl nitro carbamoyl)-d-glucosamine) has been proposed to act as a diabetogenic agent due to its ability to destruct pancreatic β-islets cells. STZ-induced diabetes mellitus is associated with the generation of reactive oxygen species causing oxidative damage [8]. Diabetics and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system and thus promotes de novo free radicals generation [9].

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The disappointing results with mono therapy, especially the worsening metabolic control often seen with the oral hypoglycemic agents [10], have led to the use of combination therapy now a days.

The principle behind combination therapy should be to use drugs with different mechanisms of action. The phytochemicals identified from plants are presenting an exciting opportunity for the development of new types of therapeutics [11]. Numerous studies on combinations have proved to be more effective than the use of individual drug alone. In this context, we are interested to study the combined effect of tetrahydrocurcumin (THC) and chlorogenic acid (CGA). THC (Figure 1), the major metabolite of curcumin exhibits diverse pharmacological activities such as hypolipidemic [12], nephroprotective [13], antioxidant [14], anti-cancer [15], anti-inflammatory [16, 17] and hepatoprotective [18]. Pari and Murugan [19] were the first to report that THC possesses antidiabetic effect in diabetic rats.

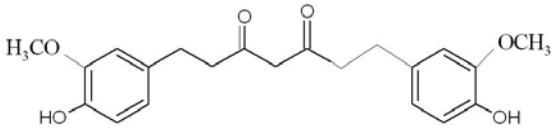

Figure 1. Structure of tetrahydrocurcumin

CGA (Figure 2) is a phenolic natural product isolated from the coffee beans, *Flos lonicerae* Japonicae. Several studies showed that caffeic acid maintain the blood glucose level in diabetes [20, 21]. The beneficial effects of coffee and CGA involve improved insulin sensitivity and enhanced insulin response [22].

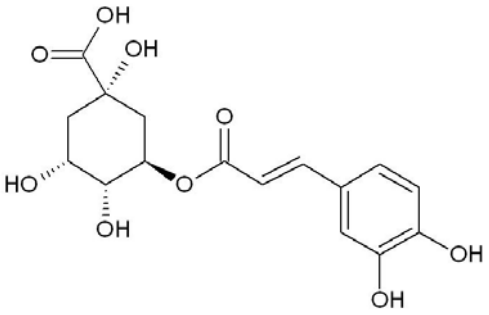

Figure 2. Structure of chlorogenic acid

In our previous studies, we have reported the combined effect of THC/CGA on rate-limiting enzymes of glycolysis and gluconeogenesis in the liver of diabetic rats [23] and also its antihyperglycemic and antioxidant effects in liver and kidney [24]. The present study was aimed to determine the combined efficacy of THC/CGA on glycoproteins in plasma, liver and kidney of normal and STZ-NA-induced diabetic rats.

Materials and Methods

Animals

Male albino Wistar rats (150-200 g) procured from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University. They were maintained in a controlled environment (12 h light/dark cycle) and temperature (28 ± 2°C). The rats had free access to drinking water and commercial standard pellet diet (Lipton India Ltd., Mumbai, India). The laboratory animal protocol used in this study was approved by the Institutional Animal Ethical Committee (IAEC) of Annamalai University in accordance with the Indian National law on animal care and use (Reg. No. 160/1999/CPCSEA; vide No. 458, 2007). The animals were acclimatized to the laboratory conditions for one week before starting the experiment.

Drugs and chemicals

Tetrahydrocurcumin (THC) was a gift sample provided by the Sabinsa Corporation, Louis, USA. Chlorogenic acid (CGA) (5-caffeoylquinic acid) and streptozotocin (STZ) were obtained from Sigma Chemical Co. (St. Louis, USA). Nicotinamide (NA) was purchased from Ranbaxy Chemicals Ltd., (Mumbai, India). All the other chemicals used in the study were of analytical grade.

Induction of diabetes

Diabetes mellitus was induced by a single intraperitoneal (i.p) injection of freshly prepared STZ (45 mg/kg b.w) in 0.1M-citrate buffer (pH-4.5) in a volume of 1 ml/kg b.w, 15 minutes after the i.p administration of NA (110 mg/kg b.w) [25]. The animals were allowed to drink 20% glucose solution overnight to overcome the drug-induced hypoglycemy. After 72 hours, plasma glucose was determined and those rats with fasting glucose levels greater than 250 mg/dL were used in the study.

Experimental design

The rats were randomly divided into five groups (each group contained 6 rats): group 1, normal; group 2, diabetic control; group 3, diabetic rats treated with THC (80 mg/kg b.w) [19]; group 4, diabetic rats treated with CGA (5 mg/kg b.w) [21]; and group 5, diabetic rats treated with THC (80 mg/kg b.w) and CGA (5 mg/kg b.w) orally using intragastric tube for 45 days.

After the last treatment, rats were fasted overnight and all the rats were anesthetized with pentobarbital sodium (35 mg/ kg, IP) and euthanized by cervical decapitation. Blood samples were collected in tubes containing potassium oxalate and sodium fluoride (1:3) for the estimation of plasma glucose and the plasma was separated for the estimation of insulin and glycoproteins. The liver and kidney were excised immediately from the animals, washed with ice-chilled physiological saline, and stored at -20°C until analyzed.

Biochemical Analysis

Determination of plasma glucose and insulin

Plasma glucose was estimated using a commercial kit method of Trinder [26]. Plasma insulin was assayed by an enzyme linked immunosorbent assay (ELISA) method using a commercial kit (Catalog No. SP-401) from United Biotech Inc., Mountain View, CA, USA.

Extraction of glycoproteins

To 0.1 ml of plasma, 5.0 ml of methanol was added, mixed well and centrifuged for 10 min at 3000×g. The supernatant was decanted and the precipitate was again washed with 5.0 ml of 95% ethanol, recentrifuged and the supernatant was decanted to obtain the precipitate of glycoproteins. This was used for the estimation of hexose and hexosamine.

For extraction of glycoproteins from the tissues, a known weight of the tissue was homogenized in 7.0 ml of methanol. The contents were filtered and homogenized with 14.0 ml of chloroform. This was filtered and the residue was successively homogenized in chloroform-methanol (2:1 v/v) and each time the extract was filtered. The residue (defatted tissues) was obtained and the filtrate decanted.

A weighed amount of defatted tissue was suspended in 3.0 ml of 2 N HCl and heated at 90°C for 4 h. The sample was cooled and neutralized with 3.0 ml of 2 N NaOH. Aliquots from this were used for estimation of hexose, hexosamine and sialic acid.

Estimation of hexose

Protein-bound hexoses were estimated by the method of Dubois and Gilles [27]. To 0.1 ml of plasma or defatted tissue

sample, 5.0 ml of 95% ethanol was added, mixed and then centrifuged. The precipitate was dissolved in 1.0 ml of 0.1 N NaOH. Subsequently,

1.0 ml of distilled water and 1.0 ml of standards (20-100 µg) were set up along with the test. To all the tubes, 8.5 ml of orcinol-sulphuric acid reagent was added and kept in a water bath for exactly 15 min at 90°C. The tubes were cooled in tap water and the colour developed was read at 540 nm against a blank.

Estimation of hexosamine

Protein bound hexosamine was estimated by the method of Wagner [28]. To 1.0 ml of plasma or defatted tissue sample was added 2.5 ml of 3 N HCl and kept for 6 h in a boiling water bath and then neutralized with 6 N NaOH. To 0.8 ml of the neutralized sample, added 0.6 ml of acetyl acetone reagent. The tubes were heated in a boiling water bath for 30 min. After cooling, 2.0 ml of Ehrlich's reagent was added and mixed well. Blank contained 0.8 ml of water. Blank and standards were processed similarly. The colour developed was read at 540 nm.

Estimation of sialic acid

Sialic acid in plasma and tissues were estimated by the method of Warren [29]. To 0.2 ml of plasma or defatted tissue, 0.5 ml of water and 0.25 ml of periodic acid was added and incubated at 37°C for 30 min. To this, added 0.2 ml of sodium meta arsenate and 2.0 ml of thiobarbituric acid and heated in a boiling water bath for exactly 6 min, cooled and 5.0 ml of acidified butanol was added. The absorbance was read at 540 nm against reagent blank.

Estimation of fucose

Fucose was estimated by the method of Dische and Shettle [30]. Two tubes containing 0.1 ml of sample (labelled as control and test) was taken. 5.0 ml of 95% ethanol was added, mixed well and then centrifuged. The precipitate was dissolved in 1.0 ml of 0.1 N NaOH. 1.0 ml of distilled water served as the blank. A series of standards in 1.0 ml volume were also set up along with the test. All the tubes were kept in ice-cold condition and 4.5 ml of H_2SO_4-water mixture was added. The tubes were kept in boiling water bath for 3 min and cooled. 0.1 ml of cysteine reagent was added to all the tubes except control and kept for 60 min at room temperature. The colour developed was read at 396 nm and 430 nm against the blank. The fucose content was calculated from the differences in the readings obtained at 396 nm and 430 nm and then subtracting the values obtained without cysteine.

Statistical Analysis

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). Values were considered statistically significant when $p < 0.05$ [31].

Results

Plasma glucose levels were significantly ($p<0.05$) increased in diabetic control rats when compared with normal rats. Diabetic rats treated with THC/CGA significantly ($p<0.05$) decreased the plasma glucose levels on comparison with diabetic control rats. A significant ($p<0.05$) decrease in plasma insulin levels in diabetic control rats was significantly ($p<0.05$) increased on treatment with THC/CGA (Figure 3).

The levels of plasma and tissue glycoproteins (hexose, hexosamine, fucose and sialic acid) are presented in table 1, 2 and 3. Significantly ($p<0.05$) higher levels of these glycoproteins were observed in the plasma and tissue of diabetic control rats when compared with normal rats. Treatment with THC/CGA to diabetic rats

Table 1: Effect of THC and CGA on plasma glycoprotein levels in normal and diabetic rats

Groups	Hexose	Hexosamine	Fucose	Sialic acid
	(mg/dL)			
Normal	93.24 ± 6.78[a]	75.16 ± 5.72[a]	27.62 ± 2.14[a]	52.62 ± 5.43[a]
Diabetic control	134.12 ± 8.92[b]	100.02 ± 6.94[b]	42.48 ± 3.82[b]	72.24 ± 6.98[b]
Diabetic + THC (80 mg/kg)	104.46 ± 7.84[c]	82.47 ± 3.72[c]	33.93 ± 2.97[c]	58.42 ± 4.95[c]
Diabetic + CGA (5 mg/kg)	106.52 ± 7.46[c]	84.42 ± 4.21[c]	32.96 ± 2.48[c]	58.04 ± 4.15[ac]
Diabetic + THC/CGA (80 + 5 mg/kg)	95.14 ± 6.82[a]	76.98 ± 5.23[a]	28.82 ± 3.02[a]	53.04 ± 5.46[a]

Each value is mean ± SD for 6 rats in each group (n=6). Values in the same column with different superscript letters (a, b, c) are significantly different ($p<0.05$, DMRT). THC: Tetrahydrocurcumin; CGA: Chlorogenic acid.

Table 2: Effect of THC and CGA on liver glycoprotein levels in normal and diabetic rats

Groups	Hexose	Hexosamine	Fucose	Sialic acid
	(mg/g defatted tissue)			
Normal	26.78 ± 2.46[a]	10.04 ± 0.82a	16.78 ± 1.32[a]	9.88 ± 0.62[a]
Diabetic control	47.94 ± 3.82[b]	18.97 ± 1.58[b]	28.06 ± 2.14[b]	4.82 ± 0.26[b]
Diabetic + THC (80 mg/kg)	31.72 ± 2.04[c]	12.05 ± 0.92[ca]	18.92 ± 1.62[ca]	6.98 ± 0.42[c]
Diabetic + CGA (5 mg/kg)	33.08 ± 2.48[c]	13.04 ± 0.89[c]	18.02 ± 1.67[ca]	7.14 ± 0.49[c]
Diabetic + THC/CGA (80 + 5 mg/kg)	28.16 ± 1.78[d]	10.54 ± 0.88[a]	16.24 ± 1.72[a]	9.02 ± 0.78[a]

Each value is mean ± SD for 6 rats in each group (n=6). Values in the same column with different superscript letters (a, b, c) are significantly different ($p<0.05$, DMRT). THC: Tetrahydrocurcumin; CGA: Chlorogenic acid.

Table 3: Effect of THC and CGA on kidney glycoprotein levels in normal and diabetic rats

Groups	Hexose	Hexosamine	Fucose	Sialic acid
	(mg/g defatted tissue)			
Normal	22.62 ± 1.58[a]	15.42 ± 1.24[a]	14.04 ± 0.86[a]	9.04 ± 0.62[a]
Diabetic control	43.04 ± 3.42[b]	31.14 ± 2.21[b]	29.12 ± 2.72[b]	4.97 ± 0.31[b]
Diabetic + THC (80 mg/kg)	28.08 ± 2.45[c]	17.92 ± 1.45[ca]	16.76 ± 0.99[ca]	6.45 ± 0.52[c]
Diabetic + CGA (5 mg/kg)	29.42 ± 2.62[c]	18.08 ± 1.38[c]	17.28 ± 1.02[ca]	6.78 ± 0.72[c]
Diabetic + THC/CGA (80 + 5 mg/kg)	24.32 ± 1.82[a]	15.18 ± 1.44[a]	14.12 ± 1.12[a]	8.92 ± 0.82[a]

Each value is mean ± SD for 6 rats in each group (n=6). Values in the same column with different superscript letters (a, b, c) are significantly different ($p<0.05$, DMRT). THC: Tetrahydrocurcumin; CGA: Chlorogenic acid.

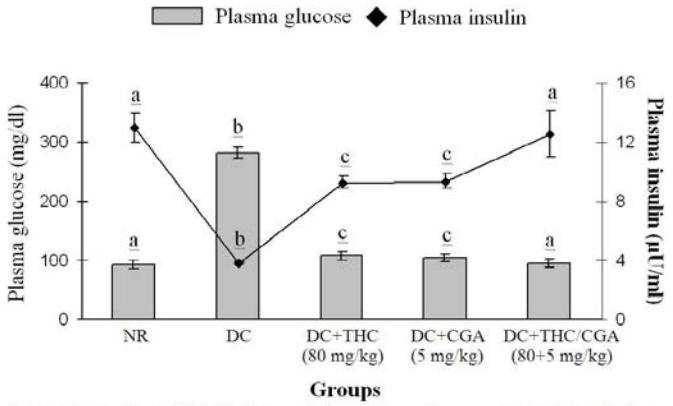

Figure 3. Combined effect of THC/CGA on fasting plasma glucose and insulin levels in normal and diabetic rats. The values are means ± SD for 6 rats in each group. Values that have a different superscript letter (a, b, c) differ significantly with each other ($P<0.05$, DMRT). NR: normal, DC: diabetic control, THC: tetrahydrocurcumin, CGA: chlorogenic acid.

for a period of 45 days resulted in a significant ($p<0.05$) reduction of hexose, hexosamine, fucose and sialic acid in plasma and sialic acid level is significantly ($p<0.05$) increased in tissues when compared with diabetic control rats.

Discussion

The currently available drug regimens for management of diabetes mellitus have certain drawbacks and therefore there is a need to find safer and more effective antidiabetic drugs [32]. The present study was aimed to determine the combined efficacy of THC/CGA on glycoproteins in plasma, liver and kidney of normal and STZ-NA-induced diabetic rats.

The mechanism by which STZ significantly increased plasma glucose and decreased insulin levels to diabetic rats is through oxidative stress. The increased levels of plasma glucose in diabetic rats were lowered by the co-administration of THC/CGA. The reduced glucose levels suggested that CGA might exert insulin like effect on peripheral tissues by promoting glucose uptake and inhibiting hepatic gluconeogenesis [33, 34], THC by its ability to scavenge free radicals and to inhibit lipid peroxidation, prevents STZ-induced oxidative stress and protects β-cells resulting in increased insulin secretion [35].

Increased glycosylation of various proteins in diabetic patients had been reported earlier [36]. In this study, we have observed altered levels of hexose, hexosamine, fucose and sialic acid in plasma and tissues of STZ-NA-induced diabetic rats. The increase in plasma glycoprotein components has been associated with the severity and duration of diabetes. In hyperglycemia, free amino groups of proteins react slowly with the carbonyl groups of reducing sugars such as glucose, to yield a Schiff-base intermediate (Maillard reaction). These Schiff-base intermediates undergo Amadori rearrangement to stable ketoamine derivative (fructosamine) [37]. Rahman et al. [36] have shown increased serum fructosamine concentrations in diabetic patients.

Sialic acid is widely distributed in tissues and in circulation. It is covalently bound to glycoproteins as the terminal sugar of oligosaccharide chains. Elevated levels of serum sialic acid are considered to be a good predictor of cardiovascular disease [38]. Diabetic rats had increased levels of sialic acid in the plasma and a decrease in tissues [39]. Increased levels of sialic acid reported in STZ-diabetic rats might be due to either enhanced sialic acid synthesis or decreased sialidase activity. Treatment with THC and CGA had normalized sialic acid levels in the plasma and tissues of diabetic rats, which could be due to the regulation of sialidase activity by insulin, since insulin is a more likely mediator of sialic acid changes.

Fucose (6-deoxy-L-galactose) is a characteristic constituent of many glycoproteins [40] and is a mobile component of plasma glycoproteins of physiological and pathological significance [41]. A raise in fucose levels could be due to increased glycosylation as in the diabetic state [4]. Experiments conducted in our laboratory showed elevated levels of fucose in diabetic animals [42, 39]. Treatment with THC and CGA had restored fucose level to near normal, which could be due to improved glycaemic status.

The biosynthesis of the carbohydrate moieties of glycoprotein forms the insulin-independent pathways for the use of glucose 6-phosphate. But the deficiency of insulin during diabetes produces

derangement of glycoprotein metabolism, resulting in the thickening of the basal membrane of pancreatic beta cells. The increased availability of glucose in the hyperglycemic state accelerates the synthesis of basement-membrane components, that is, glycoproteins [43]. This is because of depressed use of glucose by insulin-dependent pathways, thereby enhancing the formation of hexose, hexosamine, and fucose for the accumulation of glycoproteins [44].

In the diabetic state, deficiency of insulin secretion causes derangement of glycoprotein metabolism that result in the basal membrane thickening [45]. THC and CGA administration to diabetic rats normalized the levels of glycoproteins in plasma and tissues. Decreased hyperglycemic state with increased levels of plasma insulin observed in THC/CGA-treated diabetic rats might be responsible for the beneficial changes in glycoproteins in plasma, liver and kidney. In this context, other researchers have shown that decrease in hyperglycemia could lead to a decrease in glycoprotein levels [46]. Agents with antioxidant or free radical scavenging property may inhibit oxidative reactions associated with glycation [47]. THC/CGA, natural antioxidants are able to quench the free radicals responsible for the increased oxidative stress in diabetic rats.

Conclusion

This study showed that the combined treatment of THC/CGA exhibited beneficial effect on the synthesis of carbohydrate moieties of glycoproteins and also exerts antioxidative and antilipid peroxidative effects on STZ-NA-induced diabetic rats.

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