Antidiabetic effect of tetrahydrocurcumin and ptrostilbene on endothelial dysfunction

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ABSTRACT
Endothelial dysfunction plays a key role in the pathogenesis of diabetic vascular disease. Endothelial dysfunction is an imbalance in the production of vasodilator factors and when this balance is disrupted, it predisposes the vasculature towards pro-thrombotic and pro-atherogenic effects. This results in vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, pro-oxidation, impaired coagulation and nitric oxide production, vascular inflammation, atherosclerosis and thrombosis. Endothelial dysfunction is focussed as it is a potential contributor to the pathogenesis of vascular disease in diabetes mellitus. The active components of *Curcuma longa* such as curcumin and tetrahydrocurcumin (THC), a major colourless metabolite of curcumin also possesses antidiabetic, antiinflammatory and antioxidant activity. Pterostilbene (PTS) was found to be one of the active constituents in the extracts of the heartwood of *P. marsupium*. The water stored in tumblers made out of the heartwood of *P. marsupium* is used as a traditional therapy for patients with diabetes mellitus. In the present study, we assessed the effects of THC and PTS also its preventive qualities. Using an animal model, assessment for endothelial-dependent vasodilatation and the behavior of leukocytes were accomplished by using streptozotocin (STZ) - nicotinamide induced diabetic rats and its mesenteric microcirculation parameters. The results indicated that both antioxidants, THC and PTS, could significantly inhibit those abnormalities typically seen in endothelial dysfunctions ($P < 0.05$) in relation to their hypoglycemic and hypolipidemic properties. The THC administration showed more effective than PTS.

KEYWORDS: tetrahydrocurcumin, pterostilbene, endothelial dysfunction, diabetic mellitus, lipids

INTRODUCTION

Macro- and micro vascular disease are currently the principal causes of morbidity and mortality in patients with type I and type II diabetes mellitus. Loss of the modulatory role of the endothelium may be a critical and initiating factor in the development of diabetic vascular disease [1].

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by inappropriate hyperglycemia due to lack of or resistance to insulin. Patients with DM are frequently afflicted with ischemic vascular disease or wound healing defect. It is well known that type 2 DM causes amplification of the atherosclerotic process, endothelial cell dysfunction, glycosylation of extracellular matrix proteins, and vascular denervation. These complications ultimately lead to impairment of neovascularization and diabetic wound healing [2].

Diabetes is commonly associated with vascular dysfunction, particularly an impairment of endothelium-dependent relaxation, which is regarded as critical to the development of diabetes-induced vascular complications [2]. The potential contribution of the increased ROS to the development of endothelial dysfunction in diabetes has received a lot of considerable interest, since it interferes with the production of nitric oxide (NO), a key factor in multiple processes of the vascular functional homeostasis. As a result of this, several studies have investigated the roles of antioxidant agents and its benefits as a vasculoprotective agent in diabetic patients [3-5].

THC was one of the major colourless metabolite of curcumin. THC has been reported to exhibit the same physiological and pharmacological properties of curcumin [6]. Curcumin was rapidly metabolized during absorption from the intestine, yielding THC [7], which had shown the strongest antioxidant activity among all curcuminoids [8]. THC thought to play a pivotal role in protecting the cell membrane against lipid peroxidation, which exhibits its protective effect by means of β-diketone moieties and phenolic hydroxyl groups [9]. Several studies in experimental animals indicated that THC also preventes cancer, protect the inflammation, atherosclerotic lesions and hepatotoxicity [10-12].

PTS was found to be one of the active constituents in the extracts of the heartwood of *P. marsupium*. The water stored in tumblers made out of the heartwood of *P. marsupium* is used as a traditional therapy for patients with diabetes mellitus [13]. An aqueous extract of heartwood of P. marsupium has been tested clinically and found to be effective in NIDDM patients [14]. When administered to STZ induced hyperglycemic rats, PTS and marsupin two of the major phenolic constituents in aqueous decoction of the heartwood of *P. marsupium*, significantly decreased plasma glucose

[15]. In the present investigation we decided to evaluate the effects of THC and PTS to determine whether they can protect endothelial cells against diabetes induced oxidative stress.

MATERIALS AND METHODS

Animals

Adult male albino Wistar rats (8 weeks), weighing 180 to 200 g bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, were used. All animal experiments were approved by the ethical committee, Annamalai University and were in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, temperature of 24 ± 2o C, humidity of 45 to 64%. During the whole experimental period, animals were fed with a balanced commercial diet (Hindustan Lever Ltd., Mumbai, India) and water ad libitum.

Drugs and chemicals

THC and PTS was a gift provided by Sabinsa Corporation, USA. All other chemicals and biochemicals were of analytical grade.

Induction of diabetes

Non-Insulin dependent diabetes mellitus was induced [16] in overnight fasted rats by a single intraperitonial injection (i.p) of STZ (65 mg/kg body weight), 15 min after the i.p administration of nicotinamide (110 mg/kg body weight). STZ was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal saline. Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 h and then on day 7 after injection. The animals with blood glucose concentration more than 200 mg/dl will be used for the study.

Experimental design

In the experiment, a total of 24 rats (18 diabetic surviving rats, 6 normal rats) were used. The rats were divided into four groups of six each, after the induction of STZ diabetes. The experimental period was 45 days. Group I: Normal untreated rats. Group II: Diabetic control rats. Group III: Diabetic rats given THC (80 mg/kg body weight) in aqueous suspension daily using an intragastric tube for 45 days [17]. Group IV: Diabetic rats given PTS (40 mg/kg body weight) in aqueous suspension daily using an intragastric tube for 45 days [18].

On 45 days, all rats were anesthetized intraperitoneally (i.p.) with 60 mg/kg of pentobarbital sodium. A jugular vein and carotid artery were canulated for injection with fluorescence tracers and blood pressure readings were recorded. The rats were allowed to stabilize for 20 – 30 min following surgery before placing the animal on the stage of a fluorescence microscope. Mesenteric microcirculation was observed in vivo after iris blood perfusionflow was measured.

Analytical procedure

Blood glucose was estimated colorimetrically using commercial diagnostic kits (Sigma Diagnostics (I) Pvt Ltd, Baroda, India) [19]. Plasma insulin was assayed by ELISA using a Boehringer-Mannheim kit with an ES300 Boehringer analyzer (Mannheim, Germany). Haemoglobin was estimated using the cyanmethaemoglobin method described by Drabkin and Austin [20]. Glycosylated haemoglobin was estimated according to the method of Sudhakar Nayak and Pattabiraman [21] with modifications according to Bannon [22].

Thiobarbituric acid reactive substances were measured by the method of Fraga et al. [23]. Hydroperoxides were determined by the method of Jiang et al. [24].

Studies of mesenteric arteriolar response to vasoactive agents

To observe the mesenteric microcirculation, the abdomen was opened and the small intestine was displaced to expose a segment of the mesentery. A well-vascularized mesenteric window was selected and spreaded out flat over a small plexiglass platform. The mesentery was kept warm and moist by continuous superfusion with Krebs- Ringer-bicarbonate-buffered solution at 37°C and was observed under a fluorescence videomicroscope (Nikon) with a x20 objective lens. Microvessels were labeled with 5% FITC-dextran 250 (Sigma, St.Louis, USA) which was injected into the jugular vein (0.2 ml). After preconstriction arterioles with norepinephrine (NE; 10-5 M, sigma chemical Co., St. Louis, MO, USA), the vasodilatation of arterioles with acetylcholine (Ach; 10-5 M; sigma chemical Co., St. Louis, MO, USA) and sodium nitroprusside (SNP; 10-5 M; Sigma chemical Co., St. Louis, MO, USA) was conducted by using intravital fluorescent videomicroscope. The changes of the mesenteric arteriolar diameters, before and after each vasodilator was administered, were recorded real time throughout the experiment with a black and white video monitor (Sony, GM-1411 QM) and a silicon intensified target television camera (Nikon-SIT 68, Tokyo, Japan) mounted on a fluorescence microscopy using a x20 objective lens and a x10 eyepiece (CFI Plan Fluor). Video images of microvessels were stored on videotape (Sony, DX-E 120, Tokyo, Japan) connected to a video timer. Diameter of mesenteric microvessel images (20 - 40 *u*m in diameter) was measured using the software Global lab image II, (Data Translation, USA). The arteriolar diameter was calculated by averaging 3 measurements obtained from 3 different video frames using the same reference point as a marker for measuring each vessel in each frame. Arteriolar diameters were measured 5 min after Ach or SNP was administered. Vasodilatation responses were expressed as the % of maximal relaxation after preconstriction norepinephrine (NE; 10-5 M).

Evaluation of leukocyte adhesion

In order to assess the number of leukocyte adherence, the mesenteric tissues were prepared similarly to that described earlier. Instead of using FITC-dextran 250, rhodamine 6G (Sigma, St. Louis, USA) was used for labeling the leukocytes [5,25]. 0.3 ml rhodamine 6G (conc. 0.3 mg/ml; Sigma, St. Louis, USA), a total of 0.09 mg rhodamine per animal, was injected into the rat's jugular vein. Based on the rhodamine video image of each experiment, leukocytes were regarded as adherent when the cells remained stationary for more than 30 s. For each rat, 2 or 3 single vessels were selected from the iris microvascular network to assess the number of leukocytes adhering to the vessels. The number of leukocytes adhering to the venules' endothelium (20 -50 um in diameter) was countedby using the software Global lab image II.

Iris blood perfusion

The iris blood flow perfusion was measured by using a laser Doppler flowmeter (model ALF 21, Japan) with a fiber optic needle probe (wavelength 780 nm; 1 mm diameter). The needle probe was fixed 1 mm above the iris and perpendicular to the tissue. The blood perfusion was measured at 8 different locations within the iris tissue at a time and the averaged value was used for each rat.

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 if $p < 0.05$ [26].

Results

Table 1 shows the level of blood glucose, total haemoglobin, glycosylated haemoglobin and plasma insulin of different experimental groups. There was a significant elevation in blood glucose level, whereas plasma insulin levels decreased significantly in STZ diabetic rats, compared with control rats. Administration of THC tended to bring blood glucose and plasma insulin towards normal. In our previous studies of THC at 80 mg/kg was significantly better than 20 and 40 mg/kg, therefore the 80 mg/kg body weight was used in this study. The effect of THC was more prominent when compared with PTS. The diabetic control rats showed a significant decrease in the level of total haemoglobin and significant increase in the level of glycosylated haemoglobin. Oral administration of THC and PTS to diabetic rats significantly restored total haemoglobin and glycosylated haemoglobin levels. In the case of control rats, the level of haemoglobin and glycosylated haemoglobin remained unaltered.

Table 2 shows the concentration of thiobarbituric acid reactive substances and hydroperoxides in serum of control and experimental rats. There was a significant elevation in serum thiobarbituric acid reactive substances and hydroperoxides during diabetes, when compared to the corresponding control group. Administration of THC and PTS significantly decreased the lipid peroxidation in diabetic rats. The THC showed better effect than PTS.

Table 3 demonstrates the level of serum total cholesterol (TC), lipoproteins and the activity of HMG-CoA reductase in control and experimental rats. The levels of TC, low-density lipoprotein-cholesterol (LDL-C), very low-density lipoprotein-cholesterol (VLDL-C) and hepatic HMG-CoA reductase activity were significantly increased whereas the levels of high density lipioprotein – cholestrol (HDL-C) were significantly decreased in diabetic control rats. Administration of THC and PTS to diabetic rats the decreased levels of TC, LDL-C, VLDL-C levels and the activity of HMG-CoA reductase along with significant increase in the level of HDL-C.

Endothelium-dependent relaxation

Endothelium-dependent relaxation was determined by measuring the mesenteric arteriolar responses to topical application of Ach (10-5 M). The changes of arteriolar dia- meter in each group were reported in % of change from baseline diameter. In table 4, the results showed that there was a significant decrease in arteriolar responses to Ach and not to SNP in diabetic rats when compared to the controls ($P < 0.05$). Interestingly, both THC and PTS could significantly increase arteriolar responses to Ach caused by diabetes ($P < 0.001$). It is worthy to mention that THC appears to have a more potent antioxidant effect in the preservation of the endothelium than PTS. Ach-induced vasodilatation was higher in the THC group (21.41 + 4.38) when compared to PTS (18.18 + 3.15) treated groups. Moreover, the results also showed that THC (24.12 + 4.54) was able to increased SNP-induced vasodilatation higher than STZ (18.45 ±3.25), while PTS appears no difference.

Changes in iris blood perfusion

Table 5 shows iris blood flow perfusion measured in all diabetic groups (Con = 57.65± 4.35, STZ + nicotinamide = 33.52 ±2.48, STZ-THC = 47.55 ± 3.65, STZ-PTS = 42.24 ± 3.33). It was shown that iris blood flow perfusion in diabetic rats was significantly lower than the controls. However, it is possible that THC is increased in tissue perfusion due to diabetes ($P < 0.05$).

Leukocyte adhesion to endothelium

Based on the fluorescence video image, the number of leukocytes adhering to mesenteric venules is presented in table 6. At week 8, leukocyte adherence was found to be significantly greater in STZ untreated rats compared to the controls ($P < 0.001$), STZ-PTS and STZ-THC ($P < 0.05$).

DISCUSSION

Our results demonstrated that THC and PTS have potential effects in preventing diabetes-induced endothelial dysfunction which are characterized by the impairment of Ach-activated vasorelaxation and the increased leukocyte-endothelial cell interaction. Interestingly, the results showed that THC and PTS could significantly increased in diabetes. These findings seem to be the first *in vivo* evidence for demonstrating the potential of THC and PTS in protecting endothelial cells against leukocytes adhesion in diabetic rats. In our study, we also confirmed THC and PTS to have antioxidant, hypoglycemic and lipid lowering properties.

The administration of THC and PTS to decrease the increased blood glucose concentration to normal glycemic concentration is an essential trigger for the serum to revert its normal homeostasis during experimental diabetes. THC has the ability to trigger the proinsulin synthesis and also insulin release, which might be helpful to reduce the plasma glucose and increase insulin during diabetes.

Several studies have shown increased lipid peroxidation in clinical and experimental diabetes [27-29]. The results showed elevation of lipid peroxidation in the tissues of diabetic group. The increase in oxygen free radicals in diabetes could be due to rise in blood glucose levels, which upon autoxidation generate free radicals. STZ has been shown to produce oxygen free radicals [30]. Lipid peroxide mediated tissue damages have been observed in the development of type I and type II diabetes mellitus [31]. Previous studies have reported that there was an increased lipid peroxidation in serum of diabetic rats [32,33]. Our study shows that administration of THC and PTS significantly decreased the serum TBARS and hydroperoxides. transferase important in preventing lipid peroxidation in diabetic cataract rats [34].

Glycosylated haemoglobin was significantly increased in diabetic control rats, and this increase is directly proportional to fasting blood glucose [35]. Anemia is much more common disease in type 2 diabetic patients, contributing to the pathogenesis of diabetic complications. In the present study, the decreased concentration of haemoglobin indicates the anemia in STZ diabetic rats, in as much as during diabetes, the excess glucose transport in the blood reacts with haemoglobin to form glycosylated haemoglobin.

Studies have reported an increase in serum thiobarbituric acid reactive substances and hydroperoxides concentration in STZ induced diabetic rats, when compared with the normal rats. In diabetes, hypoinsulinaemia increases the activities of the enzymes, fatty acyl coenzyme and coenzyme A oxidase, which initiates β-oxidation of fatty acids resulting in lipid peroxidation [36,37]. Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity, and changing the activity of membrane-bound enzymes. Its products (lipid radicals and lipid peroxide) are harmful to the cells in the body and are associated with athrosclerosis and brain damage [37].

Our study shows that administration of THC and PTS significantly decreased the serum thiobarbituric acid reactive substances and hydroperoxides. It has been also supported by previous report of THC/curcumin increase hepatic GSH levels and induces certain forms of GSH transferase important in preventing lipid peroxidation and detoxification of toxic lipid aldehydes in diabeteic cataract rats [34].

In our experiment, on week 8, it was clearly visible that there was a significant increase in leukocyte adhesion in the diabetic rats compared to the controls. This enhancement in leukocyte-endothelium interaction was also observed in the diabetic rat's cerebral microcirculation [5]. Interestingly, the present study showed that THC and PTS were able to reduce diabetes-induced leukocyte adhesion to the endothelium. However, it has been reported that THC is a more potent antioxidant [38] because of its unique ability to scavenge oxygen free radicals better than other antioxidant agents and as a result, will significantly decrease oxidative stress, especially in endothelial cells.

There are several studies indicating a reduction of nitric oxide (NO) in the endothelium of diabetic vessels [39]. The decrease in NO may be a major contributing factor in causing a decrease in iris blood perfusion but enhancing leukocyte adhesion. The mechanisms of NO in leukocyte regulation and recruitment remains unclear even though it has been reported that it could inhibit leukocyte adhesion [40].

CONCLUSION

The study results suggest that, THC and PTS are effective in protecting the function of endothelial cells against diabetes-induced dysfunction. THC and PTS significantly reduce the level of serum lipids and lipid peroxidation marker, which are actively raised in STZ diabetic rats. THC and PTS have beneficial effect on plasma insulin and blood glucose level. Moreover it was a prevention of lipid metabolism defects could represent a protective mechanism against the development of atherosclerosis. The effect of THC was more potent than PTS.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

Table 1. Effect of THC and PTS on the levels of blood glucose, plasma insulin, haemoglobin and glycosylated haemoglobin in normal and experimental rats

Groups	Fasting blood glucose (mg/dl)	Plasma insulin (μU/ml)	Total haemoglobin (g/dl)	Glycosylated haemoglobin (mg/g Hb)
Normal	97.41± 6.38[a]	12.31± 0.73[a]	12.31± 0.75[a]	0.30± 0.02[a]
Diabetic control	279.47± 8.39[b]	3.95± 0.21[b]	8.42± 0.45[b]	0.75± 0.04[b]

Diabetic + THC (80 mg/kg)	115.62± 7.78^{c}	9.25± 0.57^{c}	11.54± 0.63^{c}	0.37± 0.01^{c}
Diabetic + PTS (40mg/kg)	133.21± 8.21^{d}	8.14± 0.45^{d}	10.14± 0.51^{d}	0.45± 0.03^{d}

Values are given as mean ± S.D for 6 rats in each group.
Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT).

Table 2. Effect of THC and PTS on the levels of TBARS and hydroperoxides in serum of normal and experimental rats.

Groups	Normal	Diabetic control	Diabetic + THC (80 mg/kg)	Diabetic+PTS (40 mg/kg)
TBARS Serum (mmoles/dl)	0.20± 0.02^{a}	0.45± 0.02^{b}	0.23± 0.01^{c}	0.28± 0.01^{d}
Hydroperoxides Serum (x 10^{-5} mmoles/dl)	10.88 ± 0.62^{a}	23.59 ± 1.35^{b}	13.58 ± 0.73^{c}	15.59± 0.75^{d}

TBARS : Thiobarbituric acid reactive substances.
Values are given as mean ± S.D for 6 rats in each group.
Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT).

Table 3. Effect of THC and PTS on changes in the levels of lipoproteins and cholesterol in normal and experimental rats

Groups	Normal	Diabetic control	Diabetic + THC	Diabetic + PTS
Serum				
Total cholesterol (mg/dl)	95.21 ± 6.55^{a}	185.41 ± 13.24^{b}	111.45 ± 8.71^{c}	125.45 ± 8.42^{d}
HDL-C (mg/dl)	55.61 ± 4.20^{a}	29.31 ± 2.45^{b}	50.41 ± 3.73^{c}	43.45 ± 3.55^{d}
LDL-C (mg/dl)	31.51 ± 2.50^{a}	128.28 ± 8.52^{b}	45.41 ± 4.41^{c}	60.42 ± 5.28^{d}
VLDL-C (mg/dl)	10.54 ± 1.20^{a}	21.21 ± 1.51^{b}	14.20 ± 1.03^{c}	16.29 ± 1.31^{d}
Hepatic HMG-CoA Reductase^{A}	1.69 ± 0.1^{a}	1.09 ± 0.1^{b}	1.60 ± 0.1^{c}	1.52 ± 0.1^{d}

^{A} – HMG-CoA / Mevalonate ratio
Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at (DMRT).

Table 4. Measurements of endothelial-dependent vasodilatation for the controls

Groups	% of arteriolar diameterAch	% of arteriolar diameter SNP
Normal	4.39 + 0.41^{a}	9.58 + 1.25 ^{a}
Diabetic control	1.28 + 0.30^{b}	10.58 + 2.58^{b}
Diabetic + THC	21.41 + 4.38^{c}	24.12 + 4.54^{c}
Diabetic + PTS	18.18 + 1.15^{d}	18.45 ±3.25^{d}

Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at (DMRT).

Table 5. Iris blood flow perfusion measurements for the Controls

Groups	Iris blood flow perfusion (A u)
Normal	57.65± 4.35^{a}
Diabetic control	33.52 ±2.48^{b}
Diabetic + THC	47.55 ± 3.65^{c}
Diabetic + PTS	42.24 ±3.33^{d}

Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at (DMRT).

Table 6. Number of leukocytes adhering to the endothelium calculated from mesenteric video images obtained from each group

Groups	Number of Leukocyte adhesion (Cell/10um of vessel length)

Normal	1.21 ± 0.12^{a}
Diabetic control	5.83 ± 0.42^{b}
Diabetic + THC	1.41 ± 0.11^{c}
Diabetic + PTS	1.89 ± 0.13^{d}

Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at (DMRT).

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