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Apigenin (4′,5,7-trihydroxyflavone) regulates hyperglycaemia, thyroid dysfunction and lipid peroxidation in alloxan-induced diabetic mice

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Abstract

The potential of apigenin (4′,5,7-trihydroxyflavone) in regulating hyperglycaemia, thyroid dysfunction and lipid peroxidation (LPO) has been revealed. While in alloxan-treated diabetic animals, a significant decrease in the concentrations of serum insulin, thyroxine (T_4) and triiodothyronine (T_3), with a parallel increase in serum glucose and hepatic glucose-6-phospatase (G-6-Pase) activity, was observed, administration of 0.78 mg kg^{-1} of apigenin for 10 consecutive days increased the levels of serum insulin and thyroid hormones with a parallel decrease in glucose concentration and hepatic G-6-Pase activity. Alloxan-induced elevation in serum cholesterol was also reduced by the compound. With respect to LPO, while in alloxan-treated animals an increase in hepatic LPO and a decrease in the activity of cellular antioxidants, such as catalase (CAT) and superoxide dismutase (SOD), and in glutathione (GSH) content was observed, administration of apigenin to alloxan-treated mice reversed all these changes, suggesting its hepatoprotective potential. Similar effects of apigenin were also observed in most of the parameters in normoglycaemic animals. It appears that apigenin has a potential to regulate diabetes mellitus, as well as disease-induced thyroid dysfunction and lipid peroxidation.

Introduction

Diabetes mellitus (DM) is one of the most common endocrine disorders. Broadly there are two types of DM, insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). The terms type I diabetes and type II diabetes are often used interchangeably for IDDM and NIDDM, respectively. Diabetes is commonly characterized by an inability of pancreatic β-cells to produce physiologically appropriate amount of insulin. This can be experimentally induced in animal models by destroying β-cells using some chemicals, including alloxan (Takasu et al 1991; Szkudelski 2001, Kannur et al 2006). So far the most common treatment for type-1 diabetes is insulin therapy.

With respect to the regulation of diabetes mellitus, several plant extracts have been investigated (Marles & Farnsworth 1994; Mukherjee et al 2006; Babu et al 2007; Valcheva-Kuzmanova 2007). Some phyto-chemicals, including epicatechin, quercetin, myricetin, pueranin, kaempferol, naringin, hesperidin, proanthocyanidins and genistinin, are also known to regulate hyperglycaemia (Chakravarthy et al 1982, Shisheva & Shechter 1992; Ong & Khoo 1996; Hsu et al 2003, De-Sousa et al 2004; Jung et al 2004; El-Alfy et al 2005; Lee 2006). However, nothing much has been studied in relation to apigenin despite the fact its presence has been demonstrated in *Allium cepa, Apium graveolens*, *Ocimum sanctum, Striga orobanchioidies, Matricaria recutita, Perilla frutescans* and *Cephalotaxus sinensis* (Sharma et al 1977; Ko et al 1991; Hiremath et al 2000; Kelm et al 2000; Nakazawa et al 2003, Li et al 2007) and some of these are also reported to be antihyperglycaemic in nature (Lans 2006; Hannan et al 2006; Li et al 2007). In fact, most of the investigations made so far on apigenin are restricted to anti-carcinogenic, anti-inflammatory, anti-fertility, antidepressant, cardioprotective and hepatoprotective properties (Wei et al 1990, Ko et al 1991; Birt et al 1997; Breinholt et al 1999; Galati et al 1999; Hiremath et al 2000; Nakazawa et al 2003; Singh et al 2004; Patel & Shukla 2007). Although, on one hand, an association exists between hyperglycaemia and thyroid problems (Ganong 1995, Panda & Kar 2006) and, on

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the other, flavonoids are believed to be antiperoxidative in nature, no systematic investigation is available on apigenin in the regulation of thyroid dysfunction and diabetes mellitus. Therefore, in this study an attempt has been made to reveal the efficacy of apigenin (4′,5,7-trihydroxyflavone) in regulating alloxan-induced type-I diabetes mellitus, thyroid dysfunction and hepatic lipid peroxidation, if any.

Materials and Methods

Chemicals

Diethylene triamine penta acetic acid, Tris buffer, sodium dodecyl sulfate, thiobarbituric acid, ethyelene-diamine-tetra-acetic acid, glucose-6-phosphate and dimethyl sulfoxide (DMSO) were supplied by E. Merck (Mumbai, India). Ellman's reagent and alloxan monohydrate were obtained from Hi-Media (Mumbai, India). Apigenin (4′,5,7-trihydroxyflavone) was purchased from Sigma (St Louis, MO); radio immunoassay (RIA) kits for the estimation of total serum Insulin, triiodothyronine (T_3) and thyroxine (T_4) were obtained from Bhabha Atomic Research Center (BARC; Mumbai, India).

Experiments were performed using colony-bred Swiss albino male mice, 30±2 g, maintained under controlled temperature (27±1°C) and photoperiod (14 h light, 10 h dark). Rodent food (Hindustan lever Ltd, Mumbai, India) and water were freely available to all the mice. Standard ethical guidelines of the Committee for the Purpose of Control and Supervision on Experiments in Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, were followed after the approval of the Departmental Ethical Committee for Handling and Maintenance for Experimental Animals.

Experimental design

Thirty-five adult healthy mice were divided into five groups of seven each. Group I, receiving only 0.1 mL of normal saline (the vehicle for alloxan), served as control.

As the test drug, apigenin was dissolved in 0.1% DMSO (Varma et al 1987). Each mouse of group II was administered with 0.1 mL of DMSO to ascertain the effect of vehicle, if any, while those of group III were administered only with 0.78 mg kg^{-1} of apigenin. Mice of groups IV and V were fasted for 18 h and then diabetes mellitus was induced by a single injection of 120 mg kg^{-1} of alloxan monohydrate (intraperitoneally) prepared in normal saline (Cooperstein & Watkins 1981). After four days of alloxan treatment, mice in group IV, serving as diabetic control, were given distilled water, while those in group V were treated with apigenin (0.78 mg kg^{-1}, s.c.) for 10 consecutive days (Tatsuta et al 2000).

Before termination of the experiment all mice were fasted overnight and killed by cervical dislocation. Blood was collected from each one and serum was separated by centrifugation and stored at −20°C until further estimations.

Assay procedures

After exsanguinations, liver was quickly removed, cleaned and then homogenized in phosphate buffer (0.1 M, pH, 7.4) with the help of a motor-driven teflon homogenizer. The homogenate was then centrifuged at 15 000 rev min^{-1} at 4°C for 30 min to obtain a clear supernatant, which was used for the estimation of hepatic lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT) and glucose-6-phosphatase (G-6-Pase) activity and for reduced glutathione (GSH) content that are routinely done in our laboratory (Panda & Kar 2003a, b; Jatwa et al 2007).

LPO, SOD, CAT and GSH estimation

In brief, LPO was determined by the reaction of thiobarbituric acid (TBA) in which malondialdehyde (MDA), a product formed due to the peroxidation of lipids, was estimated. The amount of MDA was measured by taking the absorbance at 532 nm (extinction coefficient, $E = 1.56 \times 10^5$), using a Shimadzu UV-160 spectrophotometer. LPO was finally expressed as nM MDA formed per hour per mg protein (Okhawa et al 1979). The activity of hepatic SOD was estimated by measuring the % inhibition of the pyrogallol autooxidation by the enzyme (Marklund & Marklund 1974). One unit of SOD is defined as the enzyme activity that inhibits the autooxidation of pyrogallol by 50%. CAT activity was estimated by our routine method of Aebi (1983) and was expressed as μM of H_2O_2 decomposed per minute per mg protein. For GSH content the method of Ellman (1959) was followed and was finally expressed as μg GSH per mg protein. Hepatic G-6-Pase and protein were measured by our routine laboratory methods (Lowry et al 1951; Baginski et al 1974). While protein was estimated using Folin-Ciocalteau reagent in alkaline medium, for G-6-Pase ammonium molybodate and ascorbic acid (as reducing agent) were used. The concentration of serum glucose and cholesterol was estimated by enzymatic method using kits from Qualigens Fine Chemicals (Glaxo India Ltd, Mumbai, India).

Estimation of thyroid hormones

Total circulating T_4 and T_3 were estimated by radioimmunoassay (RIA) in serum samples using the hormone specific kits, supplied by BARC (Mumbai, India) as followed earlier (Panda & Kar 2003a, b). In brief, RIA was performed using Tris hydroxy-methyl amino methane (THAM) buffer (0.14 M containing 0.1% gelatin; pH 8.6). The antisera, specific hormone standards, radiolabelled hormones (I^{125} T_4 and I^{125} T_3) and the control sera were reconstituted with assay buffer/double distilled water as specified. The reaction mixture was composed of standard/sample, buffer, radiolabelled hormone and the respective antibody, which was incubated at 37°C (30 min for T_4 and 45 min for T_3). Incubation was terminated by the addition of polyethylene glycol (PEG). Tubes were then centrifuged at 8000 rev min^{-1} for 20 min. After decanting the supernatant, traces of liquid were removed with the help of filter paper wicks without disturbing the precipitate. Finally, tubes were subjected to radioactive counting for 1 min (counts min^{-1}) using an I^{125} gamma counter. A set of quality control sera was also run with each assay.

Insulin estimation

Insulin was estimated by radioimmunoassay using the specific RIA kit and the protocol supplied by BARC (Mumbai, India), as followed earlier in our laboratory

(Jatwa & Kar 2006b). In brief, the tubes containing 200 μL of assay buffer with 100 μL of serum samples or standard were mixed and then 100 μL of primary antibodies was added to the mixture and incubated at 4°C overnight. After incubation, 100 μL of I^{125}-labelled insulin hormone was added to the mixture, kept for incubation at room temperature for 3 h, following which 100 μL of secondary antibodies was added to the mixture. The incubation was finally terminated by the addition of 1 mL of PEG. Again samples were gently mixed and incubated for another 20 min and then tubes were centrifuged at 6000 rev min^{-1} for 20 min at room temperature. After decanting the supernatant, traces of liquid were removed with the help of filter paper wicks without disturbing the precipitate. Finally, tubes were subjected to radioactive counting using an I^{125} gamma counter. A set of quality control sera was also run with each assay.

Statistical analysis

Data are expressed as mean ± s.e.m. and for evaluation of the data, one-way analysis of variance, followed by post-hoc Student's Newman–Keuls test was used. $P<0.05$ was taken as indicating statistical significance.

Results

A significant decrease in the concentrations of serum T_4, T_3 and insulin ($P<0.001$ for first two and $P<0.01$ for insulin; Figure 1), but an increase in glucose, cholesterol and in hepatic G-6-Pase activity ($P<0.001$ for all; Table 1) was observed in mice treated with alloxan. However, these effects were reversed when alloxan-treated mice received apigenin for 10 days as shown by an increase in the concentrations of serum T_3, T_4 and insulin with a parallel decrease in serum cholesterol and glucose levels and hepatic glucose-6-Pase activity ($P<0.001$ for all as compared with the respective values of alloxan-treated mice). Following administration of apigenin to normoglycaemic mice, a significant decrease in the concentrations of serum T_4, T_3, glucose and in hepatic G-6-Pase activity, but an increase in insulin concentration was observed ($P<0.05$ for T_3 and glucose, $P<0.001$ for T_4, $P<0.01$ for insulin and G-6-Pase compared with the respective control value). DMSO treatment also decreased the serum T_4 concentration significantly ($P<0.01$ as compared with the control value).

With respect to lipid peroxidation (Table 1), in alloxan-treated mice a significant increase in LPO and a decrease in SOD, CAT and GSH were observed ($P<0.001$ for all).

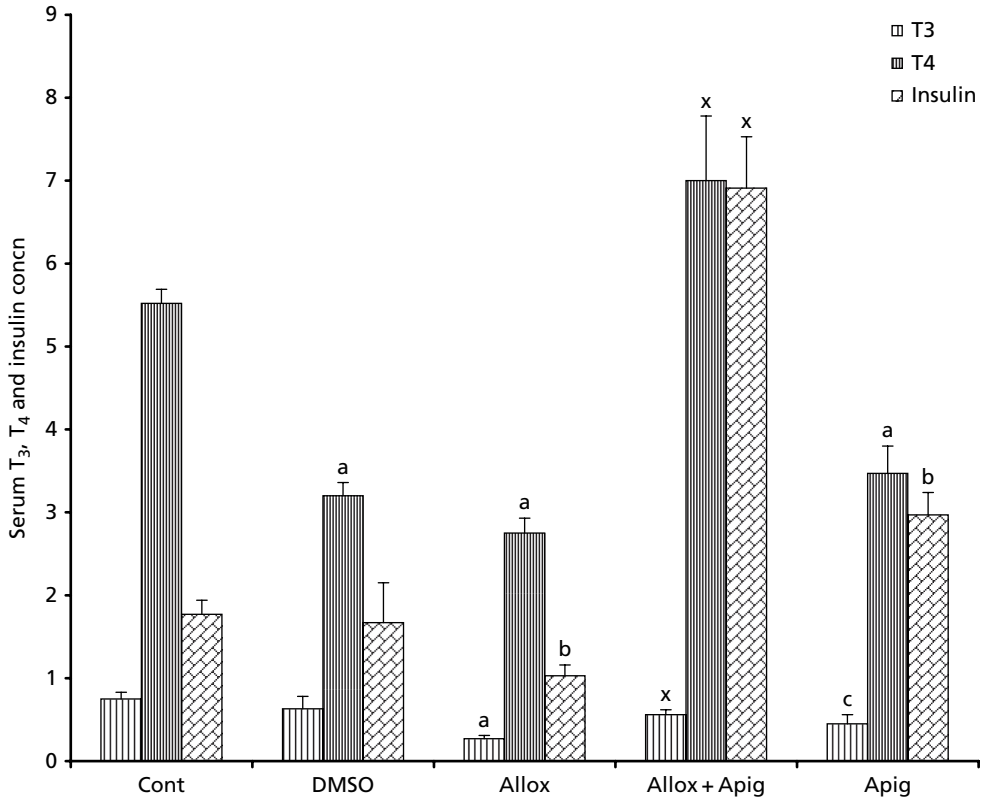

Figure 1 Changes in serum concentrations of T_3 (ng mL^{-1}), T_4 (ng $mL^{-1} \times 10$) and insulin (μU mL^{-1}) following the administration of vehicle (control), alloxan (Allox), Allox + apigenin (Apig), only apigenin or DMSO in male mice. Each bar represents the mean ± s.e.m., n = 7. Cont, Control; T_3, triidothyronine; T_4, thyroxine. $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$ compared with respective control values. $^{x}P<0.001$ compared with the respective values of alloxan-treated mice

Table 1 Effects of apigenin (API) on hepatic LPO (nM MDA formed h^{-1} (mg protein^{-1})), SOD (U (mg protein)$^{-1}$), CAT (μM of H_2O_2 decomposed min (mg protein^{-1})), GSH (μM GSH (mg protein)$^{-1}$), serum glucose (GLUC mg %), cholesterol (CHOL mg %) levels and G-6-Pase activity (μM of inorganic phosphate liberated h^{-1} (mg protein^{-1})) in alloxan (ALLOX)-induced diabetic male mice

Group	LPO	SOD	CAT	GSH	GLUC	CHOL	G-6-Pase
Control	0.996 ± 0.05	6.01 ± 0.42	54.85 ± 2.90	6.77 ± 0.58	65.84 ± 5.81	44.65 ± 3.87	2.24 ± 0.11
DMSO	0.947 ± 0.09	6.76 ± 0.39	52.17 ± 3.10	5.78 ± 0.37	75.66 ± 7.94	47.13 ± 2.67	2.27 ± 0.48
API	0.893 ± 0.06	9.70 ± 1.26^{c}	65.38 ± 2.63^{c}	12.6 ± 1.9^{c}	45.45 ± 3.99^{c}	39.94 ± 3.94	1.72 ± 0.10^{b}
ALLOX	2.92 ± 0.07^{a}	2.51 ± 0.46^{a}	30.58 ± 4.34^{a}	1.27 ± 0.98^{a}	297.7 ± 12.4^{a}	68.36 ± 4.59^{a}	4.38 ± 0.29^{a}
ALLOX+API	0.95 ± 0.09^{x}	5.37 ± 0.91^{x}	45.72 ± 2.00^{y}	6.87 ± 1.5^{x}	73.34 ± 6.24^{x}	39.77 ± 3.89^{x}	1.86 ± 0.22^{x}
API	0.893 ± 0.06	9.70 ± 1.26	55.38 ± 2.63	12.6 ± 1.9^{c}	45.45 ± 3.99^{a}	39.94 ± 3.94	1.72 ± 0.10^{b}

Data are mean ± s.e.m., n = 7. $^{a}P < 0.001$, $^{b}P < 0.01$ and $^{c}P < 0.05$ compared with the respective control value. $^{x}P < 0.001$ and $^{y}P < 0.01$ compared with the respective value of alloxan-treated mice.

However, in mice receiving alloxan along with apigenin, LPO was decreased significantly ($P < 0.001$) with the concomitant increase in SOD, CAT activity and GSH content ($P < 0.001$, 0.01 and 0.001, respectively). Mice treated with only apigenin also exhibited a significant increase in SOD and CAT activity and GSH content ($P < 0.05$ for the first two and $P < 0.01$ for the last).

Discussion

Results revealed a decrease in serum glucose concentration in alloxan-induced diabetic mice following the administration of apigenin (4′,5,7-trihydroxyflavone), indicating its potential to ameliorate diabetes mellitus. While apigenin was reported to act as an anti-cancer agent (Tatsuta et al 2000; Patel & Shukla 2007), its possible role in regulating diabetes mellitus was not demonstrated, although there are already reports indicating the anti-hyperglycaemic role of flavonoids (Stanley et al 2006; Babu et al 2007). Our findings clearly reveal that apigenin has the potential to regulate hyperglycaemia. Hepatic glucose-6-phosphatase and serum cholesterol levels, commonly elevated in diabetes mellitus (Shunkla et al 2000; Borthwick et al 2001; Shieh et al 2004), were also decreased following the administration of apigenin, further supporting its anti-diabetic property. Interestingly, alloxan-induced decrease in serum insulin level was reversed by administration of apigenin. Even in normoglycaemic mice, apigenin could enhance the insulin level, exhibiting its stimulatory action in hormone synthesis or release. These observations do suggest that the ameliorating role of apigenin in diabetes mellitus might have been mediated through stimulation of insulin secretion.

While in alloxan-induced diabetic mice, serum thyroid hormone concentrations decreased as reported earlier (Moouradian & Abourizk 1983; Baydas et al 2002; El-Shenawy & Abdel-Nabi 2006), reverse effects were observed following the apigenin administration, indicating its ameliorative role in diabetes-induced alteration in thyroid functions. Somewhat similar findings have been reported following the administration of *Cleome droserifolia* extract, containing flavonoids as active components (El-Shenawy & Abdel-Nabi 2006). Interestingly, in normoglycaemic mice apigenin was found to reduce thyroid hormone concentrations, thus exhibiting an opposite role in euthyroid mice. Although this finding corroborates the anti-thyroidal role of most of the flavonoids (Divi & Doerge 1996), in our study, the mechanism of action of apigenin appears to be different in normoglycaemic/euthyroid and in diabetic/hypothyroid mice. It is possible that apigenin may have a stimulatory role on thyroid hormone synthesis or secretion. This is easily understandable in alloxan-induced hypothyroid mice, where levels of the thyroid hormone were greatly increased. However, in normal mice, the inhibition of thyroid function could be a secondary effect. Here, as the apigenin stimulates the hormone production/secretion, after a few days, because of excess circulatory hormones, the negative feedback mechanism is initiated leading to the hypothyroid condition. Another possible cause for enhanced thyroid hormone synthesis and secretion in diabetic mice is the stimulation of insulin production (Karasu et al 1990; Jatwa & Kar 2006b). Probably because of this, apigenin could enhance insulin only to some extent (40.40%) in normal mice and to a greater extent (70.38%) in diabetic subjects. Moreover, glucose utilization is enhanced by thyroid hormone through upregulation of GLUT 4 transporters in the cell membrane, facilitating the action of insulin on glucose entry/utilization in the cell (Weinstein et al 1991; Matthaei et al 1995). Thus, in order to normalize the enhanced glucose through utilization by cells, more thyroid hormones might have been required in diabetic mice treated with apigenin.

Reactive oxygen species (ROS), such as O^{-}, OH^{-} and $H_2O_2^{-}$ radicals, normally attack the polyunsaturated fatty acids (PUFA) in the membrane lipids causing lipid peroxidation, which leads to disorganization of cell structure and function. The decomposition of peroxidized lipids yields a variety of end products, including malondialdehyde (MDA), and the imbalances between the process of forming free radicals and the endogenous defence system results in oxidative stress, which is considered to be a major reason for various diseases, including cancer, cardiac problems and diabetes (Halliwell & Gutterridge 1990). Impaired homoeostasis under induced diabetic condition is also associated with the increased production of free radicals and deficiency of the antioxidative system (Collier et al 1990). Even alloxan-induced diabetogenic activity is believed to be mediated through induction of oxygen/hydroxy free radicals and subsequent damage to the β-cells of

the pancreas (Mendola et al 1989). The underlying mechanism of alloxan action is that alloxan induces DNA damage that in turn activates the poly (ADP-ribose) synthetase and concomitantly the depletion of the cellular NAD^+ (Yamamoto et al 1981). The cellular antioxidants SOD and CAT are known to protect against alloxan toxicity (Ebelt et al 2000). Another endogenous antioxidant, GSH, a sulfhydryl reducing agent that normally assists in amino acid transport, quenches free radicals and helps to regulate the internal redox environment of cells and has been proved to bring health benefits (Orrenius et al 1984; Julius et al 1994). Thus, lipid peroxidation is counteracted by the endogenous antioxidants such as SOD, CAT and GSH. In this study, when apigenin was administered to alloxan-treated mice, it normalized the hepatic LPO as well as SOD, CAT and GSH in liver, the major organ of carbohydrate and drug metabolism. This could be the result of direct free radical scavenging activity of the flavonoid, as suggested earlier (Singh et al 2004), or might have been mediated through thyroid hormones, which are known to reduce hepatic lipid peroxidation (Maiti & Kar 1998; Jatwa & Kar 2006a). It was further supported by the observation that in normal mice, apigenin did not alter hepatic LPO significantly, whereas in diabetic mice it could reduce the same to a great extent to protect the liver from alloxan-induced peroxidation and also to maintain glucose homoeostasis.

In conclusion, it appears that the test compound, apigenin (4′,5,7-trihydroxyflavone), may have the potential to regulate diabetes mellitus/hyperglycaemia with an additional benefit of hepatic protection. However, further investigation is required for its possible use in man.

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