

Research article

**A Plant Oxysterol as a regulator of glucose homeostasis**

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**Abstract**

**Background:** Plant oxysterols like 28-homobrassinolide (HB) exhibit partial structural homology to oxysterols. HB's effect in cell metabolism was therefore investigated using rat model. **Results:** Oral administration of HB to streptozotocin induced diabetic male albino wistar rats, reduced circulating glucose level to normal level. Serum insulin content was increased by HB treatment. HB elevated the hexokinase (HK) enzyme activity of liver in the experimental animal. Peroxidative damage of cellular lipids was reduced by HB based on quantitative estimates of malonyldialdehyde (MDA) and 4-hydroxy nonenol (4-HNE) content of erythrocyte samples. **Conclusion:** The antihyperglycemic and antioxidant potential of HB was presented

**Key words:** Insulin; glucose; hexokinase; protein; receptor

**Introduction**

Oxysterols are oxidation products of cholesterol in the animal cells [1]. Enzymatic oxidation of cholesterol by Cytochrome P-450 variants (CYP7A1, CYP3A4, CYP27, CYP46s) and reactive oxygen species (ROS) have both been implicated in the generation of the different types of oxysterols found in animal cells. While CYP modified cholesterol yielded 7a, 27, 24S hydroxycholesterol that served as substrates for the synthesis of other steroids, the ROS modified cholesterol yielded 7 keto, 7betahydroxy, 5,6epoxycholesterol, and 3beta5alpha6beta dihydroxy cholestane oxysterols considered toxic to animal cells [2]. Made up of the carbon skeleton and exhibiting structural similarity to the

glucocorticoids, mineralocorticoids, and androgens, animal oxysterols were synthesized at levels far below other steroids or cholesterol [2]. Despite their low concentration, oxysterols have been implicated in the regulation of cholesterol homeostasis in animal cells, specifically capable of influencing 3-hydroxy-3-methyl glutaryl-coA reductase (HMGCoA reductase) enzyme activity, being the regulatory step in cholesterol synthesis, and for the transcriptional silencing of the sterol responsive element (SRE) [3]. Steroid signaling employed specific cytoplasmic steroid receptors for binding, followed by translocation of the steroid receptor complex to the cell nucleus. The steroid receptor complex recognized and bound chromatin regions in order to activate specific

genes for the transcriptional activity [4]. DNA sequences capable of recognizing the steroid-receptor complex functioned as the sterol responsive element, and proteins that recognized them have come to be known as sterol response element binding proteins or SREBPs. SREBPs are helix-loop-helix-leucine zipper family of proteins that required proteolytic activation and contained a transactivating domain that influenced the transcriptional activity in the cell [5]. Oxysterols in contrast, recognized and bound specific proteins localized to the nucleus called the nuclear hormone receptors. Although similar, in their structure to many of the known membrane localized receptors, specific ligands to a few of them have not been identified with certainty. They were therefore called the nuclear orphan receptors [1]. The orphan receptors were believed to be putative coactivators / corepressors of hormone-receptor complexes. Lipophilic animal cell oxysterols have been considered a transport form of cholesterol [2]. Oxysterols reportedly diminished the rate of cholesterol biosynthesis being a potent inhibitor even at 10-9 M, though cholesterol itself was weakly inhibitory at 10-5 M [6]. It is believed that the presence of a large excess of cholesterol (100 fold) in vivo reduced oxysterol binding affinity to nuclear receptors, whereas high affinity binding to receptors were noted in vitro [7]. Regulation of sterol metabolism through transcriptional and post transcriptional effects by oxysterols have been cited [2], being atherogenic in some and even apoptotic in others [1]. The identification of LXRA and LXRb nuclear hormone receptors in the regulation of cholesterol and fatty acid metabolism is taken as strong evidence to implicate a role for oxysterols in cholesterol homeostasis [8]. LXR is a close associate of the nuclear receptors PPAR, FXR and RXR. The consensual absence of an endogenous ligand for LXR has categorized this protein as an orphan receptor [9]. In recent times, a new class of polyoxygenated plant steroids has been identified called brassinosteroids. Brassinolide, dolicholide, 28-homobrassinolide, castasterone, dolichosterone, 28-dolichosterone and typhasterol

are members of this phytohormone family. Structurally, brassinolide exhibited similarity with the insect hormone ecdysteroids [10], more specifically 20-hydroxyecdysone. Natural brassinosteroids were 3-oxygenated (20b) - 5a-cholestane, 22a, 23a-diols of plant origin, that can be alkylated (C-24), methylated (C-25) and contained unsaturation (C-24- C-28). The brassinosteroid content in plants is estimated to be 10-100 µg/kg-1 in the pollen and immature seeds, and 10-100 ng/kg-1 in leaves and shoots. All the studies employing brassinosteroids have evaluated their biological potency only in plant growth. Different types of brassinosteroids were known to employ different receptor sites for exhibiting their bioactivity [11]. The presence of a ganone carbon skeleton, and the structural similarity to steroids, ecdysteroids, and oxysterols led us to investigate brassinosteroid effect on a mammalian model using the isoform, 28-homobrassinolide.

### Materials and Methods

Experiments were carried out in accordance with internationally accepted ethical guidelines for the care of laboratory animals. Male albino wistar strain rats (8-10 weeks with 12 h of light and dark cycle throughout experimental period, were used for the old) housed in propylene cages under controlled temperature and hygiene conditions, investigations. The animals were provided free access to drinking water *ad libitum*. Technical grade 28-homobrassinolide used in the study was received courtesy of Dr.Vyas, Godrej Agrovvet, Mumbai, India. Streptozotocin, BSA, Glucose and all other chemicals used were of analytical grade purchased from Sigma-Aldrich St. Louis, USA. Double distilled water was used for preparation of all reagents.

Twenty four animals were used and each group contained six animals. Experimental diabetes was therefore induced in rats by administering streptozotocin (60 mg/kg bw) intravenous to independent groups that had been fasted for 16 h [12]. Streptozotocin administered animals were maintained for 3 days to establish diabetes, registering blood glucose content 198 mg/dl.

Hypoglycemia that occurred within 24 h was prevented by feeding a 5% glucose solution orally to the diabetic rats. Diabetic animals did not become inactive or slip into coma. Control animals were maintained normal and had a blood glucose content of 89 mg/dl. Four groups of animals were identified and designated as follows  
Group I: normal control

Group II: normal rat treated with HB

Group III: diabetic control

Group IV: diabetic rat treated with HB

Following induction of diabetes, 28-homobrassinolide 50 µg (333 µg/kg.bw) in 95% ethanol and 95% ethanol alone was administered to test and control rats respectively. The dose was administered by oral route for 15 consecutive days. Blood was collected from the orbital sinus from each animal at the end of the treatment, allowed to clot and the serum was separated by centrifuging at 3000 x g and at room temperature for 5 min.

### **Biochemical parameters**

Blood glucose was estimated by the method of Asatoor and King [13]. The total cholesterol was estimated by the method of Zak et al [14]. Triglycerides were enzymatically hydrolyzed to glycerol and free fatty acids prior to estimation [15]. The chylomicron, VLDL, and LDL were precipitated by addition of phosphotungstic acid and magnesium chloride, centrifuged and the supernatant fluid containing HDL- fraction was assayed using the cholesterol reagent [16]. Each sample was estimated for total protein content by the method of Lowry et al [17]. Measurement of plasma insulin level was carried out using radioimmunoassay employing guinea pig antirat insulin primary antibodies [18].

### **Lipid peroxidation**

Packed erythrocytes were obtained by centrifuging the blood sample at 4000 x g for 15 min at 4°C, followed by washing the cells with three times physiological saline. The erythrocyte MDA concentration was determined using the method of Jain et al [19] based on thiobarbituric acid reactivity. The erythrocyte 4-HNE concentration was determined employing packed erythrocytes using the Kinter method [20].

### **HK Enzyme activity**

Tissue hexokinase/ glucokinase enzyme activities phosphorylated glucose for utilization through glycolysis or HMP-shunt metabolic pathways. Tissue hexokinase/glucokinase activity was therefore estimated using a coupled enzyme assay employing 25 mM D-glucose, 5 mM of each of ATP, MgCl<sub>2</sub> and NADP in 0.05 mM Tris-glycine buffer, pH 7.6, to which 150 µl of each tissue soluble supernatant were added. The total volume of the assay cocktail was made 2 ml. Reaction was initiated by the addition of NADP and was monitored immediately for 5 min. The sample absorbance at 340 nm was noted every min for duration of 5 min. A unit of enzyme activity was considered as the amount of enzyme that catalyzed the phosphorylation of one µmol of D-glucose/min at 37°C and at pH 7.6 [21].

### **Statistical analysis**

All the values were expressed as mean ± SEM. Statistical analysis was done using SPSS 11 (Statistical package). The statistical significance of differences between the means was assessed by ANOVA. A difference at P<0.05 was considered statistically significant.

## **Result and Discussion**

The blood glucose content of HB treated rats is shown in table 1. Glucose was significantly

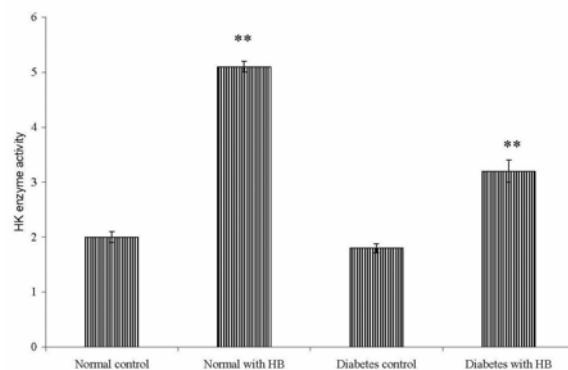
localized to the plasma membrane. Circulating glucose levels in an animal is therefore a result of

**Table 1:** Effect of 28- homobrassinolide on the biochemical parameters in streptozotocin induced diabetic rats

Sample	Glucose (mg/dl)	Serum insulin ( $\mu$ U/ml)	Cholesterol (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Normal control	89 $\pm$ 4	54 $\pm$ 2	38 $\pm$ 1.2	62 $\pm$ 3.1	16 $\pm$ 0.7	13 $\pm$ 0.4	12 $\pm$ 0.7
Normal treated	83 $\pm$ 3	56 $\pm$ 3	42 $\pm$ 2.2	71 $\pm$ 4.3	16 $\pm$ 0.9	15 $\pm$ 0.7*	14 $\pm$ 0.4
Diabetes control	198 $\pm$ 4	35 $\pm$ 1.5	34 $\pm$ 1.0	70 $\pm$ 2.6	18 $\pm$ 0.5	2 $\pm$ 0.06	14 $\pm$ 0.6
Diabetes treated	143 $\pm$ 4**	48 $\pm$ 1**	51 $\pm$ 1.9**	123 $\pm$ 2**	20 $\pm$ 0.6*	6 $\pm$ 0.3**	25 $\pm$ 1.3**

(27.7%) reduced in diabetic treated rats whereas in normal treated it is not significant (6.7%). Estimate of insulin content of serum in microunits is given in table 1. Insulin content elevated in diabetic treated rats 37.1% significantly and not significantly in normal treated rats. Determination of blood cholesterol, triglyceride, HDL, LDL and VLDL content of each blood sample yielded data as shown in table 1. Blood cholesterol increased 10.5 and 50% in normal and diabetic treated rats respectively. Triglycerides are significantly increased in diabetic treated rats (75.7%) and 14.5% in normal treated rats. HDL content not changed in normal treated rats and 25% elevation found in diabetic treated rats. LDL level significantly increased 23 and 66.6% in normal and diabetic treated rats. VLDL level increased very significantly 78.5% in diabetic treated rats and 16.6% in normal treated rats. This study suggests an antihyperglycemic effect for HB as well as an augmentation of the HDL component in rat blood. Administration of HB to experimental rats led to a reduction in MDA and 4-HNE content in the erythrocyte (Table 2). The MDA and 4-HNE content in the erythrocyte of diabetic controls were greater than that of the normal control. The restorative effect of HB on the peroxidative damage of erythrocyte was found to be statistically significant (Table 2). It is a known fact that glucose phosphorylation is the rate limiting step in glucose metabolism [22], and is catalyzed by the enzyme hexokinase [23]. Nevertheless, glucose entry into cells is facilitated by specific glucose transporters

the differences that existed between glucose mobilization from tissue glycogen, dietary glucose availability and glucose utilization by each of the tissue. Further, the rate of glucose utilization is a function of glucose entry into cells facilitated by the transporters and glucose turnover within cells through hexokinase catalyzed the phosphorylation reaction. Targeted modulation of hexokinase activity therefore greatly affected the status of glucose in the cells. In the study reported here, we have observed that



**Figure 1:** Measurement of hexokinase enzyme activity in the liver of normal and diabetic male albino wistar strain rat treated with HB orally for 15 consecutive days.

the intracellular hexokinase activity levels in the liver tissue, showed 155% and 77% increase in normal and diabetic treated rat liver compared to their respective controls (Figure 1). The data obtained suggested that HB did in fact have an impact on HK activities in these tissue and

thereby regulated the circulating blood glucose level. Ecdysteroids, a group of insect and plant steroid hormones were known to exhibit antidiabetic, antioxidant and protein synthesis inducer function in animals [24]. Our previously reported study, identified HB to have antidiabetic and antioxidant properties [25,26]. There has been considerable debate over the extent to which increased oxidative stress contributed to the development of diabetic complications. Increased membrane rigidity, decreased cellular deformability, reduced erythrocyte survival and lipid fluidity due to peroxidation of membrane lipids has been implicated in diabetes mellitus [27]. Hyperglycemia resulted in the generation of free radicals when the antioxidant defenses were exhausted within a cell and contributed to the disruption of cellular function through oxidative damage to cell membranes through enhanced susceptibility to lipid peroxidation [28]. So far, no study examining the effect of HB *in vivo* had been made on normal or diabetic rat tissue MDA, and 4-HNE content. The increased content of MDA and 4-HNE may result due to an increase in the formation of hydroxyl radicals (OH) (Halliwell and Gutteridge, 1984) while MDA and 4-HNE was major oxidation product of

**Table 2:-** Effect of homobrassinolide on lipid peroxidation in normal and streptozotocin induced diabetic rats

Para-meters	Normal control	Normal with HB	Diabetic control	Diabetic with HB
Erythrocyte -MDA	1.5 ± 0.1	1.1 ± 0.1	2.8 ± 0.2	1.6 ± 0.1**
Erythrocyte -4-HNE	1.7 ± 0.2	1.5 ± 0.1**	3.4 ± 0.2	2.1 ± 0.2***

peroxidized polyunsaturated fatty acids. Increased MDA and 4-HNE contents were therefore, an important index of lipid peroxidation [29]. Doyotte *et al* [30] pointed out that induction of antioxidant systems may follow a first exposure decreased response to a pollutant. We demonstrate here that HB is significantly inhibitory to the generation of MDA and 4-HNE in the erythrocyte of diabetic rats. Our experimental observations suggest that dietary levels of HB potentially offered a natural beneficial protection against oxidative damage

and consequently for the alleviation of related diabetic complications.

Steroids (progesterin RU-5020, glucocorticoid RU-26988) have come to be known to reduce basal glucose metabolism in isolated fat cells. Insulin stimulated glucose oxidation is known to be inhibited by some steroid antagonists (antiprogesterone, antiglucocorticoid RU-486) [31]. In castrated animals, estradiol increased basal insulin level and the quantum of insulin binding to fat, liver and muscle cell membranes. Progesterone reportedly increased basal insulin level and decreased insulin binding to fat cells. Thus, ovarian sex steroids exhibited significant effect on insulin binding to target cells [32]. Estrogen augmented glucose metabolism whereas progesterin and androgen brought about deterioration in glucose metabolism [33]. Anabolic steroid diminished glucose tolerance while contributing to substantial increase in post glucose serum insulin concentration. It is reported that 72 h alloxinated rat exhibited higher plasma cortisone levels and increased adrenal gland/body weight ratio [34]. Changes in the steroid feed back regulatory mechanisms are implicated for this phenomenon that is further related to the hypothalamic- pituitary- adrenal cortical axis.

Studies reported here provide an insight to the most likely molecular mechanism that contributed to the build up of blood glucose despite the availability of plasma insulin. The experimental observation that HB augmented the hexokinase enzyme activity in the cells of various tissues of rat and reduced the content of circulating glucose in the diabetic rat blood, clearly suggests that the lesion in type II diabetes is not related to the inability of transporting free glucose into the cells. On the contrary, the build up of free glucose within each cell diminished further transport of free glucose into each cell perhaps by saturation of the GLUTs, resulting in the increase in circulating blood glucose level. The process of glucose phosphorylation within animal cells therefore becomes critical to the maintenance of glucose homeostasis in the animal. This observation indicated that the plant oxysterol, exhibiting structural similarities with

oxycholesterols, was also likely to be recognized by the nuclear membrane hormone/orphan receptors that normally bound cholesterol based oxysterols as their ligands. It was noted that HB was capable of (1) increasing the cholesterol level in rat tissues, (2) reduce circulating blood glucose in diabetic rat (3). Our studies suggest that glucose utilization through hexokinase dependent phosphorylation as the first step in glycolysis required an unlimiting availability of active HK enzyme within the cells. The availability of active HK enzyme seemed to exhibit a dependence upon oxysterols, most likely of endogenous origin or exceptionally of dietary origin. Although dietary oxysterols were known to be available only in very low amounts, they seem to elicit a biological response in rat tissues, probably as a result of greater affinity than of oxycholesterols for binding to the nuclear hormone/orphan receptors. In other words, HB and/or plant oxysterols in general, possibly competed with endogenous oxysterols. As there had been no reports on the level of oxysterol in diabetic rat cells, it may be presumed that the endogenous oxysterols were quite available to the diabetic rat cells, but that the HK enzyme activity in diabetic rat cells remained incapable to reduce the circulating blood glucose level. In contrast, the rate of clearance of blood glucose in the diabetic rat following HB dose indicated that the phosphorylated glucose was rapidly utilized by the diabetic animal cells. The antihyperglycemic potency employed for the purpose was a mere 50 µg of HB administered in a single dose.

Our report exemplifies the significant role of hexokinase in the regulation of glucose homeostasis, and directly implicates oxysterols in general and in particular to have a specific role in the uptake of glucose and regulation of glucose homeostasis in animal cells. The importance to consider a role for the nuclear hormone/orphan receptors becomes more significant in the light of observations reported here, and demands to investigate the specific signaling mechanisms that communicated oxysterol function to culminate in specific gene expression related to the glucose homeostatic control.

## Conclusion

The antihyperglycemic property of HB was presented by reducing blood sugar and enhancing serum insulin levels. In addition, hexokinase enzyme (key enzyme in glucose utilization) activity was also elevated by this compound. HB increased cholesterol, triglycerides, HDL, LDL and VLDL in normal and diabetic rats. The antioxidant property of HB was indicated by reducing lipid peroxidation (MDA, 4-HNE) in diabetic rats.

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