Original Article

28-Homocastasterone: A Dietary Plant Keto Oxysterol Positively Modulating Hexokinase mRNA Expression and Catalytic Activity in Diabetic Male Rat

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Abstract

Background and Aim: 28-homocastasterone (28-HC) is a keto oxysteroid comes under the brassinosteroid family phytohormone. 28-HC exhibited structural similarity with mammalian oxysteroid such as OH-cholesterol. Humans were exposed to 28-HC through diet and herbal-based medicine. Therefore, in the present study, we investigated the influence of 28-HC on hexokinase catalytic activity and mRNA expression in liver, kidney, and testicular tissues in normal and diabetic male rat. **Methods:** Induction of diabetes was achieved by single peritoneal injection of streptozotocin (60 mg/kg bwt) followed by 28-HC (333.33 µg/kg b.wt) which was fed orally for 15 days. At 16th day, tissues were removed followed by hexokinase activity, and mRNA expression was analyzed. *In silico*, docking study was performed to 28-HC against glucokinase and hexokinase proteins carried out using docking application AutoDock 4.0 suite docking simulations. **Results:** 28-HC-treated rat tissues showed significantly elevated hexokinase catalytic activity and mRNA expression ($P < 0.05$). On the other hand, *in silico* molecular, docking study was performed to recognize the binding affinity of 28-HC to glucokinase and hexokinase proteins. 28-HC was bound to the drug binding pocket of glucokinase and hexokinase. The glide energy score is − 6.23 and − 6.43 for glucokinase and hexokinase. **Conclusion:** 28-HC has high binding affinity for both glucokinase and hexokinase equally. Upregulation of hexokinase activity resulted in cellular glycolysis. Hence, it is suggested that 28-HC supplemented diets were suitable for higher energy-related work activities in human and animals.

Keywords: 28-homocastasterone, diabetic, hexokinase, ketosteroid

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Introduction

Bioenergetics in living cells is structured in a sequential manner for the transformation of specific substrate to their end products. Several coenzyme and cofactors participate in these sequential events to facilitate such transformations in a regulated way both, the rate of reaction as well as energy transactions. Thermodynamically living systems are nonlinear, nonequilibrium states that support for living organisms whose metabolic activity is under controlled regulation. Modulatory influences on such reactions can, therefore, cause changes to physiological functions on living organisms.[1] A major exogenous factor that contributes to such a biochemical changes within living cells is in the dietary biomolecules. Dietary constituents play a major role in cell growth and functions as well as modulating the biochemical status of a cell for good or worse.[2] Thus, metabolic aberrations can be expected to occur as a result of either abnormal assimilation of normal constituent of a diet or due to the assimilation of uncommon or potent ingredients even when were made available at low abundance. The current anxiety to establish scientifically meaningful roles for the various natural products that are found as common constituents in most plant species, used for either dietary or medicinal needs as opened up a plethora of scientific investigation.[3] A variety of plant

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species have been employed to discern the biopotency of their constituents in this regards.[4] Knowledge on the dietary plant hormone action on mammalian cell metabolism will be important to understand plant hormonal influence on mammalian metabolic and genetic processes.[5]

Besides, 28-homocastasterone (28-HC) present in plant-based diet at quantified levels and humans were consumed daily. However, 28-HC biological influence had not been studied by any investigator for its effect *in vivo* on mammalian. Nevertheless, 28-HC structural deviation as a keto isoform from that of its hydroxyl homolog (aldo-isoform) endogenous steroids and oxysteroid, this compound attracted our interest to elucidate structure-related specific biological activity.[6] Seemingly, earlier studies carried out with endogenous aldo, keto, and epoxy oxysteroid influences on diabetic rat, indicated antihyperglycemic and antilipidemic effects of aldo-isomers as well as hyperlipidemic effect of keto-isomer was shown in diabetic patient. Similarly, experiment employed with 28-HC indicated antihyperglycemic, antilipidemic, and proerythrocytic potencies associated with this compound at dose levels of 100 µg/150 g bwt that less than mg/kilogram of the experimental rat.[7] Due to the wealth of reports that phytohormone signals to inter-kingdom communications,[8] this investigation focused on 28-HC effects on hexokinase transactivation influences on glucose status in diabetic condition using albino male rat as a mammalian experimental model. 28-HC present low-to-moderate levels in various parts of plant, human, and animals were exposed regularly more or less through plant-based diet.[9] However, this plant oxysterol whether positively or negatively modulating on mammalian cellular phosphorylating enzymes is unknown. Thus, in the present study, we have given attention to the investigation on the influence of dietary plant hormone brassinolide keto-isoform 28-HC on mammalian health in those regularly taking in this plant-based diet.

Materials and Methods

Materials

Chemicals used in this study were purchased from Sigma Aldrich were analytical grade (Sigma Aldrich, USA). Test compound 28-HC was gifted by Dr. V. S. Pori, NCL (CSIR), Pune, India. Primers used in this study were purchased from European Scientific Pvt. Limited, Bengaluru, India. Double-distilled water was used to prepare all the reagents.

Experimental rat

The laboratory animal used in this study male albino Wistar rats were purchased from Sri Raghavendra Enterprises, Bengaluru, India. The experimental animals handling and protocol strictly followed as per the CPCSEA guidelines. Experiment protocol approval was obtained from Institutional Animal Ethics Committee, Pondicherry University, India (Approval. No.IAEC/2013/01). Rats were housed in hygienic and free air circulation condition and allowed to freely access normal rat chows and water *ad libitum* throughout the experimental period, for acclimatization purpose rats were maintained at 25°C and given 12 hours light and dark cycle for 1 week. After acclimatization, 24 healthy and average weight 150 gm body weight, 3 month old rats were randomly divided into four groups each group contains six rats (n = 6) such as Group I: Control (50% ethanol 0.1 mL), Group II: Control + 50 µg 28-HC in 50% ethanol 0.1 mL, Group III: Diabetic (50% ethanol 0.1 mL), and Group IV: Diabetic + 50 µg 28-HC in 50% ethanol 0.1 mL.

Induction of diabetes and administration 28-homocastasterone

Diabetes induction achieved by a single peritoneal injection of STZ (60 mg/kg b.wt in 0.1M citrate buffer pH 4.5) was made at overnight fasted rats. After 48 h, blood glucose level was checked with the help of glucometer (Accu-Chek). The blood glucose >250 mg/dl were considered to be diabetic and used in this study. Groups II and IV were treated with 28-HC 333.33 µg/kg b.wt (50 µgm) orally for 15 consecutive days and Groups I and III served as control received 50% ethanol.

Tissue homogenate preparation

On the 16th day, rats were killed and liver, kidney, and testis were immediately removed and washed with phosphate buffer (0.1M, pH 7.4), stored at −80°C until used for biochemical assay. About 1 g of tissue homogenized in phosphate buffer with the help of tissue homogenizer (Teflon tissues homogenizer). The homogenates were centrifuged at 8000 rpm for 30 min at 4°C in Eppendorf centrifuge. The supernatant obtained from the centrifugation was used for enzymes assay and protein estimation.

Analysis of hexokinase activity

The supernatant of 10% tissues homogenates was used for the enzymes activity measurement. The hexokinase activities were analyzed employed by standard methods.[10] The tissue total proteins were estimated by the Lowry method.[11]

Hexokinase gene mRNA expression analysis

Liver, kidney, and testis total RNA isolated using TRIzol reagent protocol was followed as manufacture guidelines. The cDNA was synthesized using AMV reverse transcriptase and total RNA (100 ng) used as a template and followed by cDNA used template to amplified using specific primers, Forward, 5' GGCTGAGAGGAGACCCTTCG 3' and Reverse, 3' CCAGGTCGAACTTGAATCAT 5' nucleotide pair. The polymerase chain reaction (PCR) profile Step 1: T 94°C for 10 min, Step 2: T 94°C for 45 s, Step 3: T 72°C for 30 s, Step 4: T 72°C for 1 min, Step 5: go to step 2 for 30 cycles, and Step 6: T 72°C for 10 min. PCR amplified products were then separated by 2% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The polynucleotide band intensities were measured by densitometry.[12]

Bioinformatics – *In silico* studies

Protein preparation

Three-dimensional crystal structures of hexokinase (PDB ID: 4FOE) and glucokinase (PDB ID: 1V4S) were retrieved from RCSB protein databank (http://www.rcsb.org). Retrieved

crystal structures (both glucokinase and hexokinase) were prepared for further molecular docking analysis using PyMol software (Schrodinger LLC, Cambridge, USA). Water molecules and other nonprotein molecules removed from the glucokinase proteins employed by PyMol. Followed by polar hydrogens and Koliman charges were added to the proteins using AutoDock software 4.0.[13-15]

Ligand preparation

28-HC (CID: 5487654) were obtained from PubChem compound database (http://pubchem.ncbi.nlm.nih.gov/). The retrieved ligand 28-HC were prepared and optimized by means of ligand preparation script in AutoDock 4.0 program. The retrieved 28-HC SDF. SDF format converted to.mol2 file format using Open Babel software version 2.4.1. The ligand (28-HC) was prepared for docking as follow detecting root, torsion tree was set and saved as.pdbqt file format.[13-15]

Grid box preparation

Grid generation was carried out using the prepared crystallographic structures of both hexokinase and glucokinase. The ligand molecules 28-HC in the complexes of both protein structures were picked to form grid, therefore, the centroid of this ligand molecule in complex structures was chosen to generate grid points $X = 60$, $Y = 60$, and $Z = 60$ axis was set for further docking analysis. The grid file was generated by means of "grid generation panel" in AutoDock software version 4.0.[13-15]

Molecular docking analysis

Molecular docking analysis was carried out using docking application AutoDock 4.0 suite used to carry out the docking simulations. Different docked conformations were obtained and the conformations with strongest binding affinity toward the binding cavity of proteins were selected as the possible binding conformation for further interaction analysis. The final evaluation of protein–ligand binding was done with Glide score (docking score).[13-15]

Statistical analysis of data

The experimental data were analyzed using Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

Oral administration of 50 µg 28-HC (333.33 mg/kg b. wt) for 15 consecutive days caused elevation of tissue hexokinase catalytic activity and mRNA expression in liver, kidney, and testicular tissues in experimental rat. A 5.6% of liver hexokinase activity increased in normal treated rat, whereas 5.8% was noted to be increased in diabetic-treated group [Table 1]. In case of normal treated rat kidney enzyme, 2.2% increase in enzyme activity against the control and 1.2% that of diabetic treated rat kidney. In a similar manner, assessment of testicular tissue hexokinase activity yielded the following results, a 1.12% and 1.20% increased enzyme activity in normal and diabetic treated rat compared to control. However, hexokinase activity in diabetic liver tissue raised 10% compared to normal control. In the case of diabetic rat, kidney tissue enzyme activity reduced 50% compared to control.

Table 1: Hexokinase activity in normal, diabetic, and treated rat tissue, IU×10²/mL

Group	Liver	Kidney	Testis
Control	20±1.2	12±1.6	08±1.4
C + 50 µg 28-HC	28±1.8*	18±1.4*	14±1.8*
Diabetic	22±1.0	08±1.0	10±1.0
D + 50 µg 28-HC	26±1.6*	14±1.5*	12±1.2*

*$P<0.05$ is considered to be significant. Each group consists of six rats and each samples analyzed by triplicate. Values are expressed mean±SD. SD: Standard deviation

Hexokinase gene mRNA expression was analyzed employing PCR, using a set of hexokinase gene-specific primer. The specific nucleotide sequence of hexokinase gene generated by PCR reaction was stained with ethidium bromide and band intensity quantified by using densitometric software in gel documentation system. The amplicon band intensity of a series of selective tissue hexokinase was as follow. Hexokinase gene expression in liver yielded 28.90% above in 28-HC-treated diabetic rat compared to diabetic control and 27% above in normal-treated group [Figure 1a and b]. Hexokinase gene expression in kidney yielded similar band size, whereas the difference in the band intensity for the diabetic and normal 28-HC-treated group bands was only 13.44% and 21.20% higher than the respective control. Using the same primers, the hexokinase gene amplicon of testicular tissue yielded 8.4% above in normal-treated group than control. The 28-HC-treated diabetic rat testicular tissue hexokinase gene band intensity was 22.60% greater than diabetic control. In all cases [Figure 1b], the hexokinase band size exhibited 400 bp size.

In silico study

In the present *in silico* studies, molecular docking was performed to examine the binding affinity and favorable binding interaction of the 28-HC (ligand) molecule with the enzymes. Crystal structure of the human glucokinase (PDB ID: 1V4S) and crystal structure of human hexokinase (PDB ID: 4FOE) as a target proteins for docking analysis. Dietary plant keto oxysterol 28-HC (CID: 5487654) a ligand compound for docking analysis with both enzymes. Before docking analysis, ligand molecules were prepared by means of OpenBabel and AutoDock software. Followed by this, well-prepared keto oxysterol 28-HC was docked into the catalytic cavity of both enzymes. Different docked conformations generated for ligand molecule (28-HC) and docking conformations which having lowest glide score was selected as a most probable conformation. The carefully chosen docking conformation of 28-HC with target proteins glucokinase and hexokinase was taken further analysis binding mode of interaction. Three-dimensional crystal structures of the proteins and ligand molecule are shown in Figure 2. The binding modes of 28-HC in the catalytic cavity of glucokinase and hexokinase are shown in Figures 3 and 4.

Interaction analysis between ligand molecule and glucokinase proteins

Docked complexes of 28-HC exhibited glide score −6.23 kcal/mol, indicating good binding affinity toward target protein glucokinase.

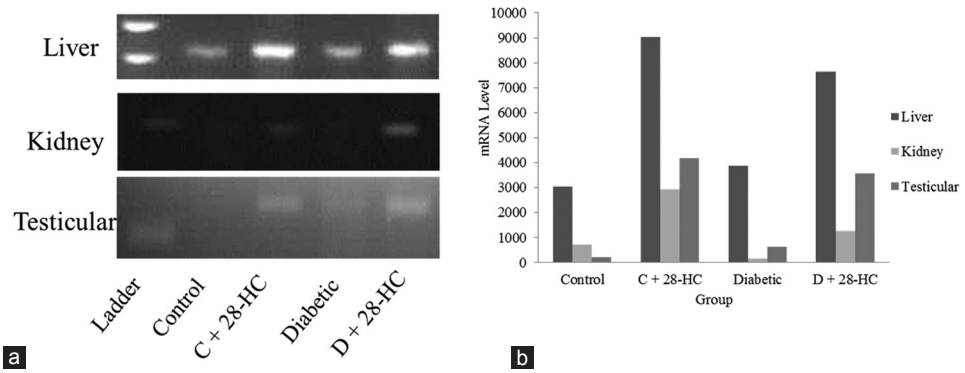

Figure 1: Hexokinase mRNA expressions in 28-homocastasterone-treated rat tissue. (a) HK PCR product band. (b) Hexokinase mRNA expression

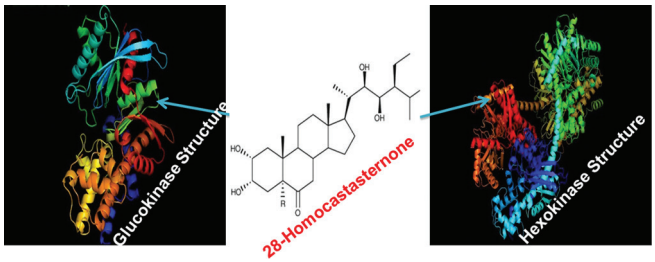

Figure 2: Proteins and ligand

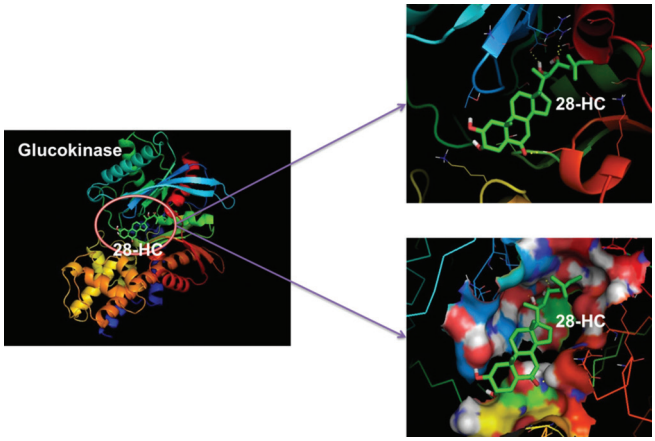

Figure 3: 28-homocastasterone binding cavity on glucokinase

The complex of glucokinase with 28-HC [Figures 3 and 5] shows strong binding interaction with ligand binding cavity of the enzyme by forming H-bonded interactions with residues Asp78, Asp78, Thr82, Arg85, Ser411, and Ser445 and the docking complex of glucokinase with 28-HC revealed that Thr82 in structural component a chain involved in hydrophobic interactions with a bond length 3.99Å [Table 2].

Interaction analysis between ligand molecule and hexokinase proteins

The G-score of 28-HC exhibited glide score −6.43 kcal/mol, suggesting that keto oxysterol 28-HC has a good binding affinity toward hexokinase, compared to the glucokinase enzyme, indicating good binding affinity toward target protein glucokinase [Table 2]. The complex of hexokinase with 28-HC [Figure 4] shows strong binding interaction with ligand binding cavity of the enzyme by forming multiple nonbonded interactions such as hydrophobic interactions with residues Arg53, Leu247, Glu249, and Ala796. The docking complex of protein with 28-HC [Figure 6] shows that polar residue Asp793 in structural component B chain involved in H-bond interaction with a bond length 3.48Å.

Discussion

The present study investigated the influence of phyto keto oxysterol on tissue hexokinase. 28-HC was present at low-to-moderate levels in various parts of the plant. Humans and animals were exposed regularly more or less through plant-based diet.[9] However, this plant oxysterol whether it has a positively or negatively modulating effect on mammalian cellular phosphorylating enzymes were unknown. Earlier studies with 28-HC exhibited antiglycemic potential associated with improved glucose utilization through glycogen biosynthesis pathway activation in diabetic rat.[16] Dietary and circulating mobilized glucose are utilized by glycolysis, glycogenesis, and pentose phosphate pathways in mammalian cells. In all these pathways, the first step was glucose were trapped inside the cells in the form of glucose-6-phosphate that was catalyzed by one of the phosphorylating enzyme hexokinase, further glucose-6-phosphate utilized by various metabolic pathways.[17] Hence, cellular glucose utilization depends on the rate of glucose phosphorylation by phosphorylating enzyme activity. Therefore, we studied the influence of 28-HC on phosphorylating enzyme hexokinase catalytic activity in diabetic patient. Earlier study employed with brassinosteroid family aldo isomer 28-homobrasslinolide indicated that it influenced on hexokinase activity positively in male rat tissues.[18] However, the 28-HC was also affecting this enzyme catalytic activity and mRNA expression in diabetic-treated rat. Nevertheless, enzyme activity and gene expression were reduced in diabetic rat. Thus, suggestive that 28-HC improves glucose utilization through hexokinase enzyme inductive potency in the diabetic rat. Clearly, therefore, it can be asserted that 28-HC exhibits differential roles in the different tissues under normal and abnormal status of health. However, earlier studies also indicated that the brasslinosteroid aldo isoform 28-homobrasslinolide induced rat hexokinase gene expression

Table 2: 28-homocastasterone interaction with glucokinase and hexokinase protein

Protein	G-score	H-band	H-band distance	Hydrophobic	Polar	π-π
Glucokinase (1V4S)	−6.23	Asp78, Asp78, Thr82, Arg85, Ser411, Ser445	3.71, 3.59, 2.98, 3.04, 2.91, 2.58	Thr82	-	-
Hexokinase (4FOE)	−6.43	Asp793	3.48	Arg53, Leu247, Glu249, Ala796.	-	-

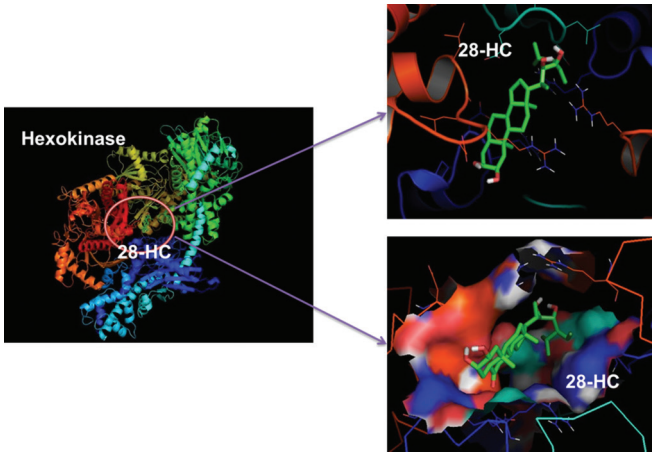

Figure 4: 28-homocastasterone binding cavity on hexokinase

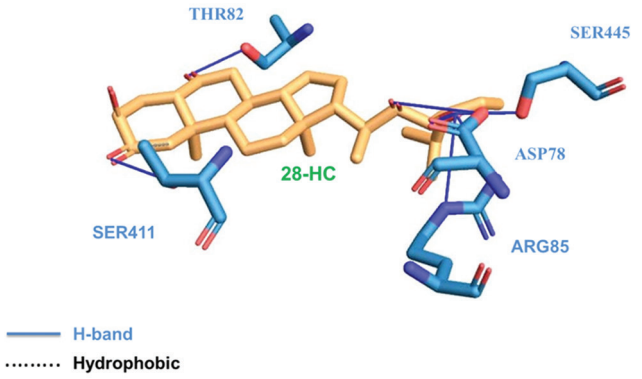

Figure 5: Glucokinase amino acids interaction with 28-homocastasterone

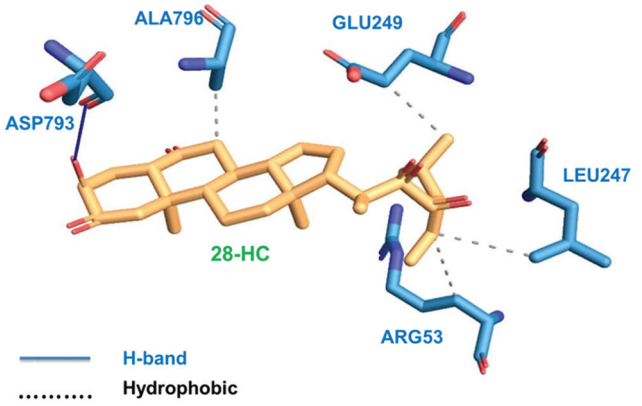

Figure 6: Hexokinase amino acids interaction with 28-homocastasterone

through the transcriptional regulation Muthuraman and Srikumar.[19] The increase in tissues hexokinase activity and mRNA expression observed in the liver, kidney, and testicular tissue indicates that a similar signaling cascade is in the process in rat tissues as a response to 28-HC administration to the experimental animal. The docking analysis of ligand molecule with both glucokinase and hexokinase confirmed that the keto oxysterol 28-HC have good binding affinity with the target similar study conducted by Pandurangan M *et al.* and Angadi *et al.* with natural compound guggultetrol.[13,15] Interaction analysis confirms that the keto oxysterol 28-HC shows similar binding nature of natural ligands guggultetrol and glucose with glucokinase and hexokinase by forming H-bond and nonbonded interactions with the ligand binding cavity residues, suggesting that 28-HC can modulate the enzyme catalytic function and protein structure preservation.

Conclusion

Dietary plant ketosteroid 28-HC transactivates hexokinase gene expression in tissues and thereby resulting in cellular glycolysis. Hence, it is suggested that 28-HC supplemented diets were suitable for higher energy-related work activities in humans and animals.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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