Chemico-Biological Interactions 277 (2017) 8–20

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

28-Homocastasterone down regulates blood glucose, cholesterol, triglycerides, SREBP1c and activates LxR expression in normal & diabetic male rat

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ARTICLE INFO

Article history:
Received 3 May 2017
Received in revised form
9 August 2017
Accepted 15 August 2017
Available online 16 August 2017

Keywords:
28-Homocastasterone
Blood glucose
Cholesterol
Triglyceride
Liver x Receptor

ABSTRACT

Plant steroids are being recognized as influential secondary bio factors, assimilating in animal tissues through diet and affecting their cellular metabolic function to varying degree. They modulate catalytic and signaling functions in mammalian cells, affecting cellular homeostasis. The effect of phyto brassinosteroid ketoisoform 28-homocastasterone (28-HC), was assessed for its influence on blood glucose, plasma lipid and selective signal marker levels in normal and diabetic male wistar rat models. A 15 day oral feed regimen employing the experimental rat, noted that circulating blood glucose, cholesterol and triglyceride level in diabetic rat were markedly reduced by this compound. This study confirmed that the keto form had anti-hyperglycemic and anti-lipidemic potency associated with it and was available to man and animals in their diet. Western blots of marker protein, PCR amplicons of marker mRNA expressions and In Silico studies suggested that 28-HCeffect is being mediated through LxR molecular operatives in the rat cell.

1. Introduction

Search for natural products to control disease states in man has opened up a plethora of approaches for the identification of the perfect drug molecule. Among the natural products, plant steroids attract a vital interest due to their bio-potency in plant seed germination, growth, flowering, immune function and stress tolerance. Their ubiquitous presence in plant varieties and their assimilation into mammalian tissues through diet and folk medicine makes them potent secondary factors available to mammalian cells capable of modulating cellular metabolic processes. Polyoxygenated plant sterols belong to the brasslinolide (BS) family of phytohormones involved in regulating plant vital functions. 28-homocastasterone is a ketoisoform member of this family and attracted our interest for investigating its biological function in animal cell [1]. Earlier studies employing an aldo isomer 28-Homobrasslinolide (28-HB) yielded antihyperglycemic and anabolic effects in experimental rat. Tissue hexokinase and testicular marker levels of 17βHSD, 13βHSD enzymes and StAR protein expression were augmented by this compound [2]. Isomeric LxR α and β receptor expressions were also induced to suggest co-expression of the two isoforms being required to mediate testicular testosterone synthesis in rat. *In Silico* studies indicated greater avidity for 28-HB binding to LxR protein than for testosterone [3]. These findings suggested that brasslinolide members can act as natural ligands to LxR receptors that also function as nuclear transcriptional regulators. That specific activation of LxR isoforms resulted in differential responses in mammalian tissues was first recognized by Kiss E et al. [4]. Cholesterol oxidation products such as 24-hydroxycholesterol, 25-hydroxycholesterol, 24,25-hydroxycholesterol, and 24-ketocholesterol have been identified as endogenous ligands for LxR receptors. It is considered that LxR-alpha is responsible for cholesterol metabolism and LxR-beta for glucose and triglyceride homeostasis [5]. Targeting a specific LxR isoform is thus a focus for drug development and for minimizing LxR induced unwarranted effects [6]. Most prevalent hyperglycemia associated disease states are those due to diabetes and atherosclerosis, the two leading causes for cardiovascular disorder (CVD). CVD risk factors include elevated serum cholesterol and

Abbreviations: 28-HC, 28-homocastasterone; LxR, Liver x Receptor; ABCT, ATP-binding cassette transporters; SREBP1c, sterol regulatory element binding protein-1c; TNF-α, Tumor necrosis factor-α; HKI, hexokinase-I; PEPCK, Phosphoenol Pyruvate Carboxy Kinase; PC, Pyruvate carboxylase; ACC, Acetyl CoA Carboxylase.

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http://dx.doi.org/10.1016/j.cbi.2017.08.010
0009-2797/

triglyceride levels. Currently, statin based therapeutics is in use for potent lipid lowering effect in patients. Higher dosage needs and inadequate potency to lower triglyceride and cholesterol levels by statin and the need to advocate antihyperglycemic drug intake additionally by these same patients for controlled glycemic status in them leads to search for novel compounds preferably a natural one, that embodies the required biopotencies in a single compound [7,8].

This study therefore embarked on the use of the brassinolide ketoisoform 28-homocastasterone (28-HC) in experimental diabetic rat through a sub acute oral feed regimen for 15 days and noted potent antihyperglycemic and antilipidemic property co-associated with this natural product. This compound attenuated blood and tissue cholesterol and triglyceride level in diabetic rat, for any given dose. Earlier use of 28-HC [9] noted reduced ALT, AST, and LDH enzyme activity in rat plasma with increase in liver glycogen content, indicative of intervention in metabolic transamination processes, pyruvate generation and energy yield.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in this study were of analytical grade purchased from Sigma Aldrich (USA). 28-Homocastasterone was gift from Dr.V.S.Pori. National Chemical Laboratory (NCL, CSIR), Pune, India. Glucose, cholesterol and triglyceride Kits were purchased from Agape Diagnostic Pvt.Ltd, Kerala, India. Double distilled water was used to prepare all reagents in this study. The primary and secondary antibodies used in the study were purchased from the Santa Cruz Biotech (USA). LxR-alpha, LxR-beta, SREBP1c, ABCT, TNF-alpha and TMB-H_2O_2 were purchased from Bangalore Genei, India. The gene specific primers were purchased from European Genomics, Bangalore, India.

2.2. Experimental animals and protocol

Male albino *wistar* rats were purchased from authorized dealer Sri Ragavendra Enterprises, Bangalore, India. The animals were handled as per the CPCSEA guidelines (IAEC/Approval.No.2013-14/01). The animals were housed in hygienic polythene cages and maintained with free air circulation at room temperature (24 °C). For acclimation purpose, the rats was housed for one week and given normal rat chow pellet and water *ad libitum* on a 12 h light and dark cycle. The rats were divided into 8 groups, each group contain 6 rats having an average weight 150-180 gm.

The groups were,

Group I: Control (0.1 ml of 50% ethanol)
Group II: Control+28-Homocastasterone (50 μg in 0.1 ml of 50% ethanol)
Group III: Control+28-Homocastasterone (100 μg in 0.1 ml of 50% ethanol)
Group IV: Control+28-Homocastasterone (150 μg in 0.1 ml of 50% ethanol)
Group V: Diabetic control (0.1 ml of 50% ethanol)
Group VI: Diabetic+28-Homocastasterone (50 μg in 0.1 ml of 50% ethanol)
GroupVII: Diabetic+28-Homocastasterone (100 μg in 0.1 ml of 50% ethanol)
Group VIII: Diabetic+28-Homocastasterone (150 μg in 0.1 ml of 50% ethanol)

2.3. Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of 60 mg/kg bwt streptozotocin in citrate buffer (0.1 M, pH 4.5) to overnight fasted rat. After 48 h, the blood glucose level was measured with the help of a glucometer (OneTouch Horizon, Auccva check). Glucose content >250 mg/dL was considered to be diabetic and were used for experiment. Control and diabetic rats were administered 28-Homocastasterone dose by oral gavage for 15 consecutive days. On 16th day, blood glucose and plasma lipid levels were analysed. Rat tissues were collected for marker protein and mRNA studies.

2.4. Estimation of biochemical parameters

Plasma glucose, cholesterol and triglyceride were analysed using diagnostic kits (agape India Pvt.Ltd, Kerala). Plasma markers were analysed in control animals with blood drawn from orbital sinus and treated blood samples were obtained by cardiac puncture, used for analysis.

2.5. Western blots for protein expression

The LxR-α & β isoforms, SREBP1c, ABCT and TNF-α protein expression was analysed by western blot technique. In brief, liver tissues homogenate proteins were separated in 10% SDS-PAGE (Mini Protean II System, Bio-Rad, USA), and the resolved proteins were transferred onto a nitrocellulose membrane (NYTRAN, Keene, NH, USA).Protein transfer to membrane was confirmed by Ponceau S staining, and the membranes were incubated in blocking buffer (PBST buffer containing 0.1% Tween and 5% non-fat dry milk powder) for 1 h at room temperature with constant shaking. Goat/rabbit polyclonal primary antibody against LxR-α, LxR-β, SREBP1c, ABCT and TNF-α was used as the probe and blots were incubated overnight at 4 °C with constant shaking. Following a wash with PBST buffer the blots were further incubated at room temperature for 1hr with horseradish peroxidase conjugated rabbit anti-goat/goat anti-rabbit IgG (1:1000 dilution). TMB/H_2O_2 (Bangalore Genei, Bangalore, India) substrate was used to reveal antibody specific protein banding. The detected protein bands were quantified by densitometry.

2.6. Total RNA isolation and RT-PCR

Total RNA was isolated from selected rat liver tissues using TRIzol reagent protocol as per manufacturer's guidelines. Briefly, 100 mg of fresh tissue was frozen in liquid nitrogen and homogenized in a Teflon homogenizer using 1 ml TRIzol reagent. Samples were centrifuged at 14,000 rpm for 10 min at 4 °C, supernatant transferred to a new tube, and 200 μl chloroform was added to the sample and vortexed for 30sec, followed by centrifugation at 13,000 rpm for 15 min at 4 °C. The aqueous layer in the sample was transferred to new tube. RNA was precipitated by addition of the 500 μl of isopropanol, and the samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The RNA pellet obtained was washed with 70% Ethanol (three to four times). RNA pellet was resuspended in 100 μl DEPC water. The RNA integrity was analysed on a 2% (w/v) agarose gel by electrophoresis. The purity and amount of RNA was measured based on 260/280 nm absorption ratio and using the standard estimation formula. The cDNA was synthesised from isolated total RNA (100 ng each) from control and treated rat tissues, by RT-PCR employing AMV reverse transcriptase. (LxR-α)5' −GCGTCCATTCAGAGCAAGTGT-3′,3′-TCACTCGTGGACATCCCAGAT-5′, (LxR-β)5′-CTCTGCCTACATCGTGGTCATCT3-', 3′-ATGAAGGCATC-CATCTGGCAGGT-5′, (HKI) 5′-GGCTGAGAGGAGACCCTTCG-3′,3′-