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Zerumbone modulates O-GlcNAcylation in HepG2 cells in high cholesterol/high fat milieu

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Unhealthy diet can alter the state of hepatic O-GlcNAcylation, a post-translational modification of proteins driven by diet that is nutrition-driven with consequences on health. Various plant-derived bioactive compounds have demonstrated therapeutic potential in metabolic disorders. Zerumbone (Zer), a sesquiterpenoid from *Zingiber zerumbet* was earlier determined to be a potent AMPK activator. We hypothesized that targeting AMPK by Zer, O-GlcNAcylation and associated molecules could be modulated, leading to beneficial effects. HepG2 cells was used as a model to evaluate the effect of Zerumbone on O-GlcNAcylation in high fat (HF) and high cholesterol (HC) milieu brought about by Palmitic acid (PA) and water-soluble cholesterol, respectively. PA/HC led to reduced global O-GlcNAcylation, which was prevented on treatment with Zer in therapeutic mode. Associated enzymes ((Glutamine-Fructose Amino transferase-GFAT and O-GlcNAcyl Transferase-OGT)) responsible for O-GlcNAcylation were normalized to control levels that were on par with known AMPK activators metformin and 6,7-dihydro-4-hydroxy-3-(2'-hydroxy[1,1'-biphenyl]-4-yl)-6-oxo-thieno[2,3-b] pyridine-5-carbonitrile (A-769662). Zerumbone potentially normalized O-GlcNAc levels and could aid in reducing complications caused byfaulty diet by targeting AMPK.

Keywords: Plant bioactive, Zerumbone, O-GlcNAcylation, AMPK activation, Faulty diet, Metabolic disorder

Introduction

A diet rich in cholesterol/high fat results in hypercholesterolemia and brings about fat accumulation in the live[1,2]. Non-alcoholic fatty liver is one of the primary causes of metabolic syndrome. Apart from changes in fat metabolism, various molecular targets, namely, AMP-associated protein kinase (AMPK) and Hexosamine Biosynthetic Pathway (HBP), are affected[3]. AMPK a serine-threonine kinase functions as cellular energy sensor. Based on the cytosolic AMP/ATP and ADP/ATP ratio, it modulates itself to promote anabolism or catabolism[4,5].

Activation of AMPK is altered during pathological conditions such as diabetes and hyper cholesterolemia[4,6]. AMPK modulation is known to impact various other pathways. One such pathway is the HBP, also known as the Nutrient Sensor pathway[3,7,8]. The end product of the HBP pathway, Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), is involved in O-GlcNAcylation of proteins, a post-translational modification believed to play essential roles in various aspects of cell signaling, aging, immunomodulation, etc[9-11]. Our earlier study showed that rats fed with hypercholesterolemic diet had reduced pAMPK (phosphorylated AMPK)/AMPK ratio and a reduced global O-GlcNAcylation in the hepatocytes with altered lipid levels[12]. Additionally, AMPK can also get O-GlcNAcylated, altering its activity[13]. Overall, the increase or decrease of protein O-GlcNAcylation is deemed to be a marker for pathological condition. Homeostasis of O-GlcNAcylation is advocated to maintain general health[14,15].

Since both AMPK and HBP are dependent on the nutritional status of the cell it is expected that the dietary bioactives can play an equally important role in modulating them. There are quite a number of dietary bioactives which are known to modulate AMPK[16,17]. However, the impact of dietary bioactives on O-GlcNAcylation has not been determined to the best of our knowledge. We earlier showed that

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zerumbone (2,6,9,9- tetramethyl-[2E,6E,10E]-cycloundeca-2,6,10-trien-1-one), belonging to Zingi beraceae family, a monocyclic sesquiterpene was able to potently activate AMPK in a kidney cell line[18].

Since AMPK activation and O-GlcNAcylation are dependent on each other, we hypothesized that Zer (zerumbone) would be able to modulate O-GlcNAcylation and bring about beneficial effect in the context of diet induced hypercholesterolemia.

Material and methods

Chemicals

A-769662 (SML2578), Cholesterol methyl-β cyclodextrine (C4951), fat-free BSA (9048-46-8), Palmitic acid (P05000), Metformin (M0605333), chemiluminescent peroxidase substrate-1 (CSP1A60-1KT), dimethyl sulfoxide (DMSO; D8418), Thiamet G (1009816-48-1), protease inhibitor cocktail (P2714), phenylmethylsulfonyl fluoride (PMSF; 78830), anti-rabbit secondary antibody (A6154), anti-mouse secondary antibody (SAB4700977), OGA (SAB4200311) and cell culture media were obtained from Sigma, USA. Antibodies against Actin (4970), AMPK (2532), pAMPK (2535), ACC (3662), pACC (11818), O-GlcNAc (9875) and OGT (24083) and were obtained from Cell signaling technology, USA. Bovine serum albumin was from Himedia (9048-46-8), India. Precision plus protein color marker (1610374), were from Bio-Rad Ireland. PVDF membrane was from PALL Corporation, USA. All cell culture consumables were from Thermo-Scientific. All other chemicals and reagents used were of analytical grade. Zer was extracted from *Zingiber zerumbet* as per the procedure described earlier using the hydro distillation method and purity determined by gas chromatography[19].

Cell culture and treatment

HepG2 cells (depicted as "cells" throughout) were used for the studies. Cell culture and induction of hypercholesterolemia is as previously described[12]. Briefly, cells were seeded at a density of 1×10^5 cells/well and incubated for 24 h. They were then treated with palmitic acid (PA, 400 μM) and cholesterol–methyl-β-cyclodextrin (CH, 100 μM) individually to mimic hypercholesterolemic conditions.

The effect of Zer treatment on cell viability was determined by MTT assay. Cells were grown in 96-well plates (1×10^5 cells/well). Zer in varying concentration (0-50 μM) was treated to cells, and then treated with MTT solution (10 μL) and incubated in CO_2 incubator for 4 h. The Formazan crystals formed after removing the media were dissolved in DMSO and the absorbance was read at 595 nm. The concentration cut-off of 75% was considered for further studies.

For modulating AMPK, cells were treated individually with PA /CH along with Metformin, Zer, and A769662. All the dosages were decided based on its effect on cell viability (data not shown). At the end of the experiment, cells were washed twice with PBS and used for analysis.

Western blot analysis

Cell homogenate with O-GlcNAcase (OGA) inhibitor, Thiamet G were subjected to SDS-PAGE followed by WB according to the previously described protocol[12]. Data is expressed in normalized fold change with β-actin as loading controls. All antibodies were diluted as per manufacturers instructions.

Statistical analysis

Values are expressed as mean ± SD. GraphPad Prism 8 software was used for data analysis. Statistical analysis is shown between the groups using 1-way ANOVA with Tukey post – t test and considered statistically significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

The purity of Zer was assessed by GC (Fig. 1). Results revealed that Zer used in our experiments was 99% pure.

Schema of experimentation

Experiments were performed to determine the effect of Zer in HC and HF milieu in both therapeutic and curative mode in HepG2 cells as per the schema given (Fig. 2). The HC or HF milieu was induced by treating the cells individually with either cholesterol-methyl-β-cyclodextrin (CH) or Palmitic acid (PA), respectively. PA at 0.4 mM and CH at 100 μM were chosen for our studies based on the results obtained with respect to cell toxicity studies (Data not shown).

Effect of Zer treatment on AMPK activation

Initially effect of Zer in various concentrations was determined for cell toxicity. Dose at which 75% of cells survive was chosen for further experiments (Fig 3a). Effect of Zer in varying concentrations were determined for activation of AMPK in milieu of CH

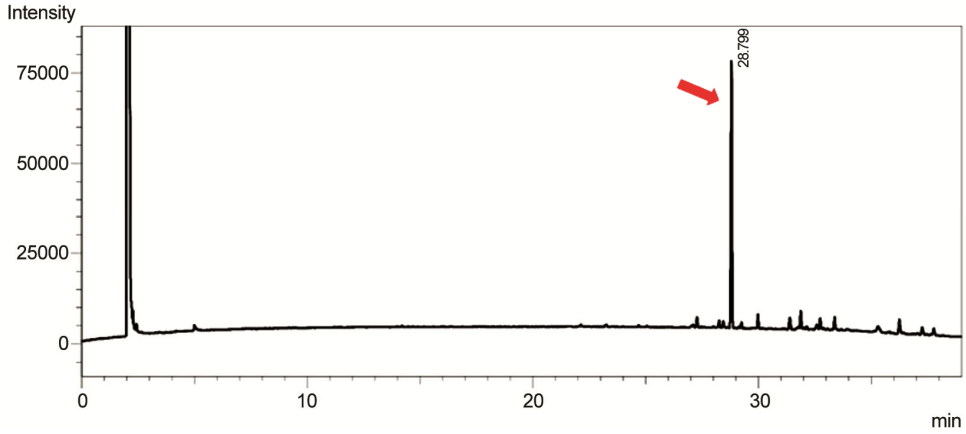

Fig. 1 — GC Analysis of Zerumbone. Zerumbone was extracted from Z. zerumbet and analysed as detailed in "Methods". Peak at 28.7 min was identified as Zerumbone based on the elution of standard Zerumbone.

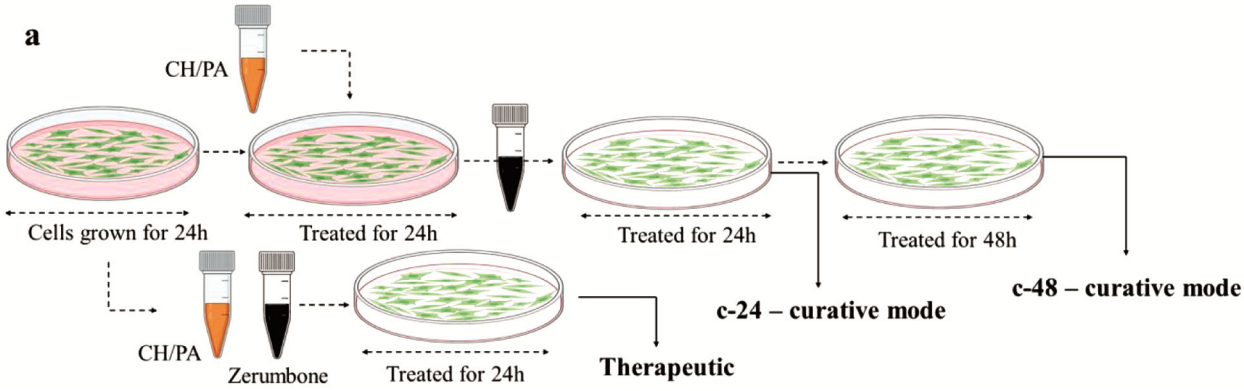

Fig. 2 — Pictorial representation of the experimental plan.

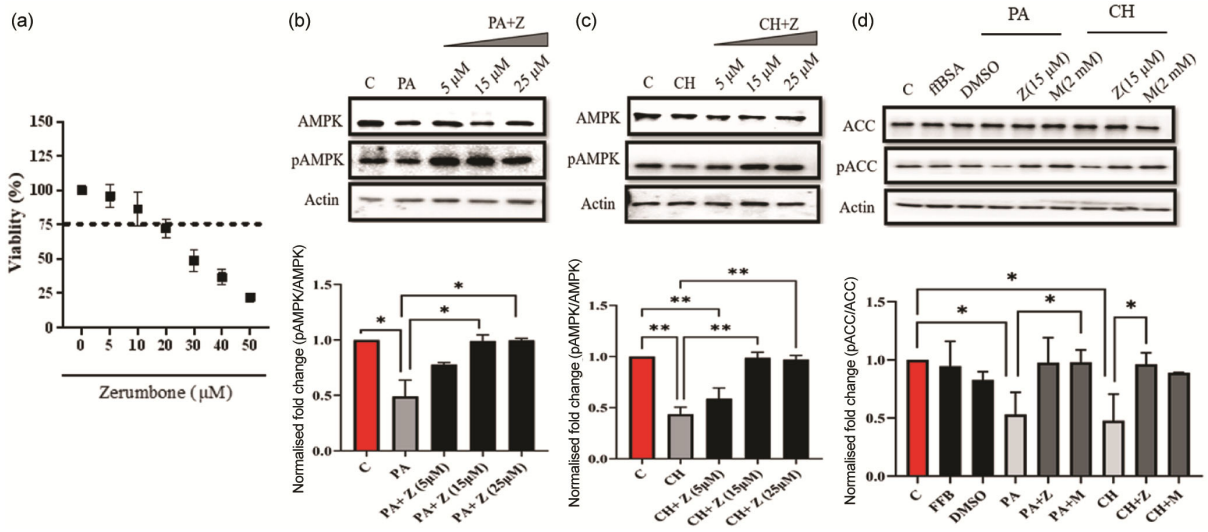

Fig. 3 — Zerumbone leads to cellular AMPK activation in HepG2 cells. Effect of Zer at tested concentrations on cell viability (Dotted line shows 75% viability) (a). HepG2 cells were treated with either PA or CH, with varying concentrations of Zer (0-25 µM) for 24 h, and analyzed for pAMPK/AMPK (b and c). ACC phosphorylation with Zer and Metformin (d) Values are represented as mean ±SD (n= 3 independent experiments). Data is given as normalised fold change against β-actin as loading control. Statistical significance is given as $P \leq 0.05$ *, $P \leq 0.01$** and $P \leq 0.001$***. Abbreviations used – C- non treated control (cells only), ffBSA- fat free BSA control, DMSO- DMSO control, PA- Palmitic acid, CH- Cholesterol-methyl-β-cyclodextrin, Z-Zerumbone and M- Metformin.

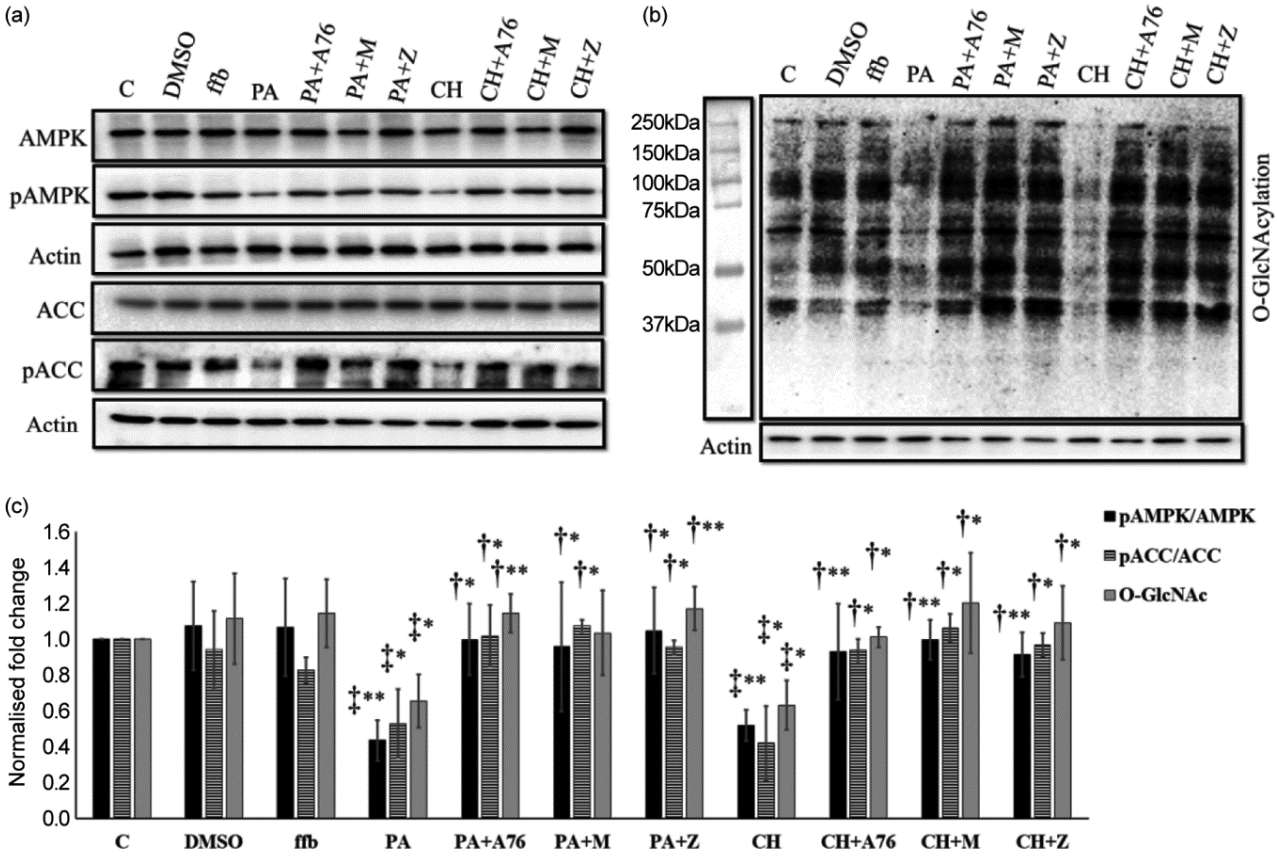

Fig. 4 — Effect of AMPK modulation on global O-GlcNAcylation. Hep G2 cells were treated with AMPK modulators (direct, indirect and Zer) as detailed in Materials and Methods and analyzed post-treatment. Blots were analyzed for pAMPK/AMPK and pACC/ACC (a), global O-GlcNAcylation (b) and quantitated by densitometric analysis (c). Associated enzymes GFAT 1 Statistical significance is given as $P \leq 0.05$ *, $P \leq 0.01$** and $P \leq 0.001$***(‡- compared to controls and †- compared to PA and CH). Abbreviations used A76 –A769662, rest as in Fig. 3.

and PA. Treatment with Zer resulted in activation of AMPK (measured in terms of phosphorylation at Thr172 α subunit, pAMPK) and was restored to control levels at 15 and 25 µM concentration in both PA- and CH-treated cells. At lower dose of 5 µM, AMPK was activated but was not significant when compared to only PA/CH-treated cells (Fig. 3b and c). Hence, further studies were carried out using Zer at 15 µM. At this concentration, Zer was also able to modulate the downstream target, the ACC (Fig. 3d), and restore it to the level of normal control cells, which was on par with metformin treatment (2 mM) without affecting the cell viability.

Effect of Zer treatment on global O-GlcNAcylation

Cells treated with PA and CH showed decreased global O-GlcNAcylation levels, which was prevented by Zer treatment for 24h. This was also the case with known AMPK activators - A-769662 (direct AMPK activator) and Metformin (indirect AMPK activator) (Fig. 4a and b). On the other hand, Compound C (CC, AMPK inhibitor) treatment decreased pAMPK and O-GlcNAcylation levels (Supplementary Fig.1)

Effect of Zer treatment on enzymes involved in O-GlcNAcylation

O-GlcNAcylation is associated with a relatively increased expression of the key enzyme of the HBP, Glutamine fructose-6-phosphate amidotrans ferase (GFAT) 1 in Zer and metformin-treated groups when compared to untreated groups, which showed a decrease in both PA- and CH- condition (Fig 4c). An increase in O-GlcNAc transferase (OGT) expression, the enzyme involved in the transfer of O-GlcNAc moiety, was observed in Zer and Metformin treated group (Fig. 5a). However, no change was observed in OGA, the enzyme involved in the removal of O-GlcNAc moiety in all the treated groups (Fig. 5b).

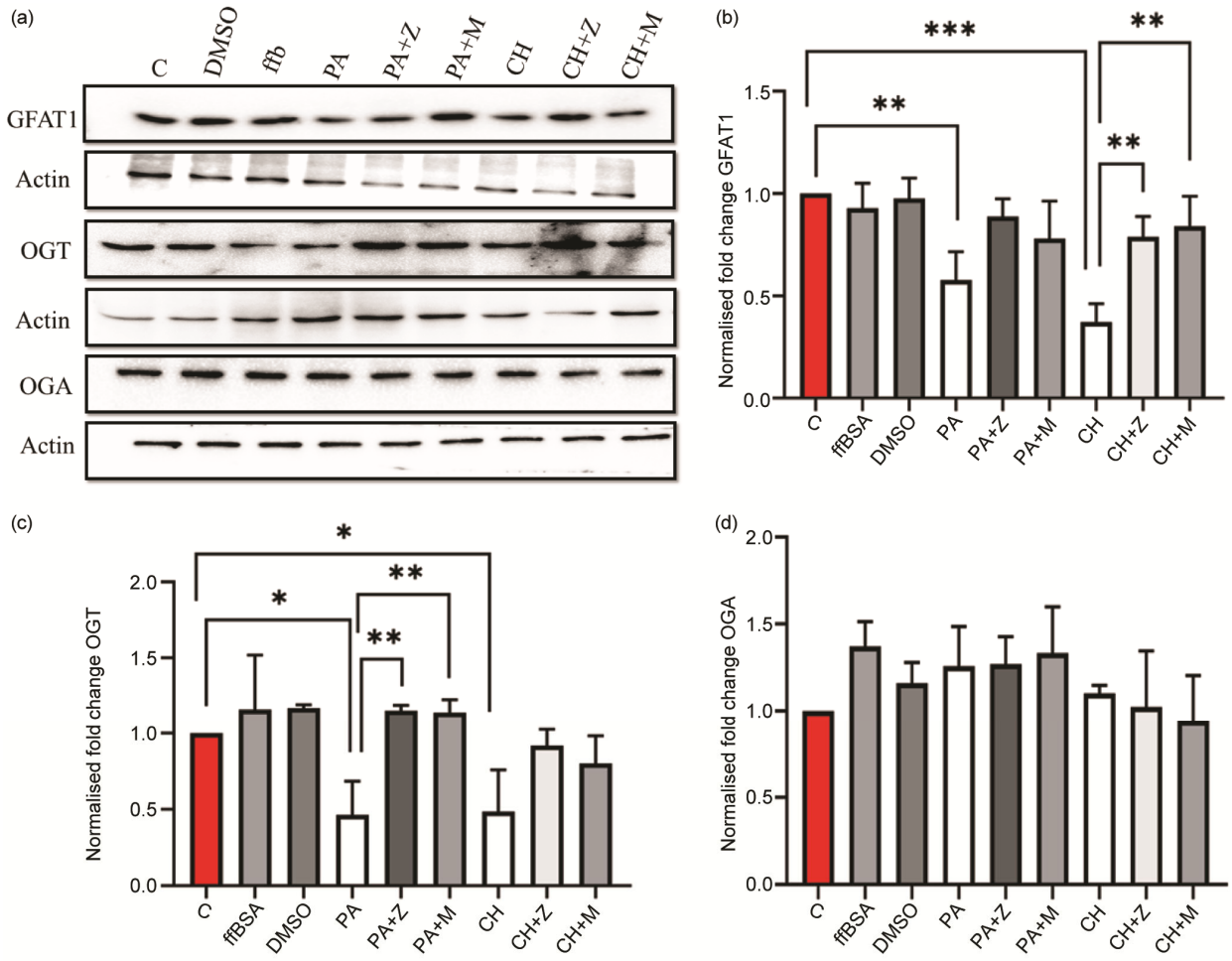

Fig .5 — Effect of Zer on PA and CH condition in curative mode. Known concentration of total cell lysate (n=3 independent expts) was anlysed for pAMPK/AMPK (a) and global O-GlcNAcylation (given as a representative), (b) and densitometric analysis, (c) Abbreviation c – curative. (d) OGT and OGA was analyzed and quantified by densitometry, Blots are given as a representative of n=3 independent experiments, Rest of the details- as in Fig.3 and 4.

Effect of Zer on AMPK activation and O-GlcNAcylation in curative mode

Since Zer treatments led to significant activation of AMPK and restored O-GlcNAcylation levels to control levels, we wanted to determine if similar observations could be made after the onset of the condition. The cells were grown to attain 60-80% confluency, after which they were treated with PA and CH for 24 h and then treated with Zer for a further 24 and 48 h (Curative mode; 'c'). Zer, however, failed to activate AMPK or restore O-GlcNAcylation in curative mode at 24 or 48 h (Fig. 5c & d).

Discussion

Increased consumption of fat/glucose disrupts the nutrient balance, contributing to the advancement of human diseases linked to nutrient excess[20]. O-GlcNAcylation responds to dietary imbalance as a nutrient sensor. Some of the plant bioactives show good potential to counteract complications associated with metabolic disorders[21]. Zer is one such known to mitigate hyperglycaemia, lipid lowering by inducing fatty acid oxidation, ameliorate high fat induced adipogenesis by increasing the SIRT1 levels, is anti-tumorigenic and has a unique anti-viral effect[22-25]. Further AMPK modulation by pharmacological agents demonstrated beneficial effect in terms of bringing in O-GlcNAcylation homeostasis in diet-induced hyperc holesterolemia[12]. In this study, Zer was able to activate AMPK and phosphorylate downstream target ACC in HepG2 cells grown under conditions which simulate high fat and high cholesterol. This is in concurrence to our earlier reports wherein we

had shown that Zer from wild ginger is able to activate AMPK under high glucose condition and bring about subsequent phosphorylation of ACC in a kidney cell line, Madin Darby Canine Kidney (MDCK)[18]. HepG2 shares typical liver-specific receptors like Low-Density Lipoprotein Receptor (LDLr), Scavenger Receptor class B Type 1 (SR-B1), and many fatty acid receptors responding to free fatty acids making them a relevant model for hepatic lipid processing. The fact that Zer was able to act therapeutically indicated its ability to function as a modulator to bring about metabolic benefit, as has been the case with many other dietary bioactives[16,26].

Activation of AMPK is beneficial and is one of the therapeutic targets in the treatment of metabolic diseases such as diabetes[27,28]. AMPK activation has far-reaching effects in terms of combating oxidative stress, autophagy, mitochondrial biogenesis, inflammation, etc[29-33]. Its ability to act on HBP through GFAT is well known[3]. Phosphorylation of GFAT by AMPK is one of the mechanisms through which HBP is modulated. Conventionally, phosphorylation of GFAT by AMPK is known to inhibit HBP, leading to decreased O-GlcNAcylation[34]. In diets inducing hypercholesterolemia, a decrease in O-GlcNAcylation could be due to decreased GFAT coupled with its decreased phosphorylation of GFAT. One of the limitations have been not looking into the phosphorylation status of GFAT. However, it can be predicted based on decreased AMPK activation that the phosphorylation of GFAT could have decreased as well[13]. Zer treatment on the other hand prevented the decrease and preserves the O-GlcNAcylation to control levels likely by preventing the deleterious initiation of lipotoxicity. However, once the damage is established due to HF/HC the process becomes self-sustained. Earlier studies show that Tumor Necrosis Factor-Related Apoptosis-Inducing ligand treatment failed in counteracting palmitate-induced cytotoxicity in retinal epithelial cells in sustained presence of palmitate in the system[35]. Moreover, bioactives are more effective in preventive or early intervention. The increase and decrease in O-GlcNAcylation levels has consequences for whole-body metabolism[14,36,37]. Earlier reports on neuronal cells show that glucose-deprived conditions dramatically increase OGT and O-GlcNAcylation status coupled with increased pAMPK[38]. Further, it is also shown that AMPK governs OGT mRNA stability because transcriptional inhibition of OGT merely prevented the glucose-deprived increased O-GlcNAcylation, whereas inhibition of AMPK decreased the OGT mRNA level[3,37]. This is also supported by our findings that inhibition of AMPK using CC leads to reduced O-GlcNAcylation states. Hence, factors, dietary or otherwise which brings in O-GlcNAcylation homeostasis can help maintain proteins' structural and functional integrity.

Above results indicate that Zer can act therapeutically and can be used as a functional ingredient in bringing about O-GlcNAcylation homeostasis which impinges on the health. Further studies in animal models are warranted.

Conclusion

Zer, was able to modulate global O-GlcNAcylation in HepG2 cells therapeutically and was mediated by AMPK activation. O-GlcNAcylation modulation was associated with differential changes in expression of associated enzymes namely GFAT and OGT. Zer appears to have greater efficacy in preserving metabolic homeostasis as preventive under lipid overloads due to faulty diet rather than reversing an already established lipotoxicity.

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