FARNESOL SUPPRESSES LIPID ACCUMULATION IN HepaRG CELL BASED MODEL OF HEPATIC STEATOSIS.

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ABSTRACT

Farnesol, a 15-carbon isoprenoid alcohol, and its metabolites, collectively known as "farnesoids," modulate metabolic processes in rodent liver through activation of nuclear receptors such as peroxisome proliferator-activated receptor alpha (PPARα), constitutive androstane receptor (CAR), and farnesoid X-receptor (FXR). However, the effects of farnesoids on human hepatic lipid metabolism have not been investigated. Therefore, the aim of this study is to determine whether farnesol modulates hepatic pathways associated with human lipid homeostasis. For these studies, we selected the HepaRG cell line, a bipotent progenitor cell line that can be differentiated into "hepatocyte-like" cells. We hypothesize that farnesoids regulate the expression of genes involved in human hepatic lipid metabolism by altering the activity of the lipid-sensing nuclear receptors, PPARα, CAR, and FXR. Because CAR signaling is a phenotype that is associated with differentiated hepatocytes, we evaluated the intactness of CAR signaling as a test of the suitability of HepaRG cells for our studies. Treatment of differentiated HepaRG cells with the known CAR activators, CITCO and phenobarbital, strongly increased expression of the CAR target gene, CYP2B6, and farnesol treatment also increased CYP2B6 expression. We are currently performing studies to evaluate functionality of the FXR and PPARα pathways and to test farnesol's ability to modulate them. Additionally, the HepaRG cells are being treated with the free fatty acids oleate and palmitate or with inhibitors of microsomal triglyceride transfer protein to promote lipid accumulation in the cultured HepaRG cells. This will allow us to investigate whether farnesol could be effective for treating pathophysiological abnormalities such as hepatic steatosis.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), which is characterized by the accumulation of lipids in liver, is prevalent in almost 30% of the world's adult population and in 60–80% of diabetic and obese patients [1]. An association between NAFLD and cardiovascular disease (CVD) is becoming increasingly recognized, and several studies suggest that NAFLD is an independent risk factor for CVD [2]. Thus, pharmacological interventions that could prevent or reverse hepatic steatosis could potentially decrease the overall risk of CVD in NAFLD patients.

Farnesol is an isoprenoid that is produced endogenously from farnesyl pyrophosphate, an intermediate metabolite in the cholesterol biosynthesis pathway [3], or that can be acquired through dietary intake of plant products such as tomatoes, peaches, and strawberries [4]. Both *in vivo* and *in vitro* studies have suggested that farnesol and/or its oxidative metabolites can modulate various physiological processes including lipid metabolism. For example, in rodents, farnesol treatment lowered serum triglycerides (TG) and reduced hepatic fatty acid accumulation [4,5] through both PPARα-dependent and independent pathways. These effects were associated with decreased expression of genes involved in fatty acid synthesis and increased expression of genes involved in fatty acid oxidation. Treatment with squalene synthase inhibitors, a class of anti-cholesterol drugs that cause the accumulation of endogenous farnesoids [6], have also been shown to decrease serum and hepatic TG levels [7], demonstrating the potential of farnesol treatment to be used as a therapeutic approach against hepatic steatosis. The goal of our studies is to determine the role of the FXR, PPARα, and CAR nuclear receptors in mediating the effects of farnesol on lipid metabolism using a cell culture model of human steatosis.

METHODS

HepaRG cell culture and differentiation: HepaRG cells were obtained from Biopredic International under a Material Transfer Agreement with INSERM-Transfert (Paris, France). The cells were cultured in HepaRG growth medium (Williams' Medium E supplemented with 10% FBS, 5μg/ml insulin, 0.1M triamcinolone acetonide, and 100U/ml Pen/Strep) for 14 days followed by HepaRG differentiation medium 1 (HepaRG growth medium supplemented with 1% DMSO) for 48 hours and HepaRG differentiation medium 2 (HepaRG growth medium supplemented with 2% DMSO) for a further 14 days.

Treatment: Differentiated HepaRG cells were cultured in HepaRG growth medium containing 2% FBS for 72 hours and then treated as described in the individual figure legends, either in HepaRG growth medium with 2% serum or in serum-free medium.

Oil red O (ORO) assay: Cells were washed with PBS and fixed in formalin for 60 min. After fixing, cells were washed three to four times with water. ORO solution (5 mg ORO/ml isopropanol) was added to each well and incubated at room temperature for 10 min. Cells were rinsed three to four times with tap water and allowed to dry. To extract ORO, isopropanol was added to each well, and the plate was shaken at room temperature for 15 min. Samples were read at 500 nm using a microplate absorbance spectrophotometer.

TG measurement: Cells were pre-treated with either bovine serum albumin (BSA) or oleic acid (OA) in BSA for 24 hr and then treated with either BSA, OA, farnesol, or OA and farnesol in combination for another 48 hr. Cells were then collected into PBS and sonicated. Lysates were directly used to measure TG using the colorimetric TG assay kit from ScienCell Research Laboratories (Carlsbad, CA). TG content s were normalized to cellular protein levels.

mRNA measurement: Following treatment, RNA was extracted from cells and cDNA was synthesized. Quantitative determination of gene expression was performed using TaqMan or SYBR Gene Expression Assays and the StepOne Plus Real Time PCR machine (Applied Biosystems, Foster City, CA).

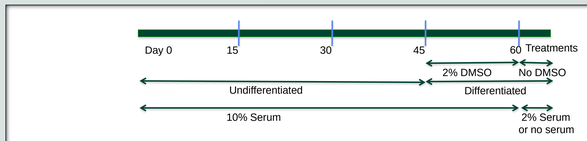

Figure 1: Timeline for HepaRG culture establishment, differentiation, and treatment.

RESULTS

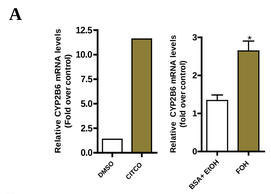

Figure 2A: Effect of CAR agonist (CITCO) or farnesol treatment on CYP2B6 mRNA levels in HepaRG cells. (Left) HepaRG cells were treated with vehicle control (DMSO, 0.1%) or the CAR agonist CITCO (0.1μM) for 48 hr. (Right) HepaRG cells were treated with vehicle control (BSA+ethanol) or farnesol (FOH, 100μM) for 48 hr. Cultures were then harvested for measurement of CYP2B6 mRNA levels, as an indication of CAR activation. Each bar represents the mean of CYP2B6 mRNA relative to control. *p<0.05 (n=3 independent experiments).

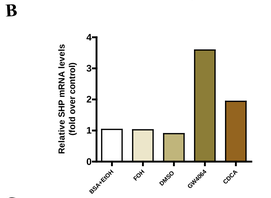

Figure 2B: Effect of FXR agonist (GW4064 or chenodeoxycholic acid, CDCA) or farnesol treatment on short heterodimer partner (SHP, NR0B2) mRNA levels in HepaRG cells. HepaRG cells were treated with vehicle control for farnesol (BSA+ethanol), farnesol (FOH, 100μM), vehicle control for GW4064 and CDCA (DMSO 0.1%), GW4064 (1μM), or CDCA (100μM) for 48 hr. Cultures were then harvested for measurement of SHP mRNA levels, as an indication of FXR activation. Each bar represents the amount of SHP mRNA relative to control.

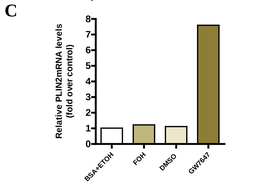

Figure 2C: Effect of PPARα agonist (GW7647) or farnesol treatment on perilipin 2 (PLIN2) mRNA levels in HepaRG cells. HepaRG cells were treated with vehicle control for farnesol (BSA+ethanol), farnesol (FOH, 100μM), vehicle control for GW7647 (DMSO, 0.1%) or GW7647 (10μM) for 48 hr. Cultures were then harvested for measurement of PLIN2 mRNA levels, as an indication of PPARα activation. Each bar represents the amount of PLIN2 mRNA relative to control.

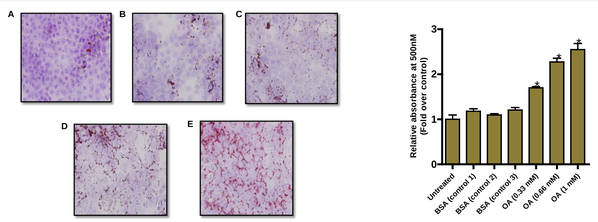

Figure 3: Oleic acid (OA) dose-dependently increases intracellular lipid accumulation in HepaRG cells. (Left) Differentiated HepaRG cells were treated with no drug (A), vehicle control (BSA+ethanol, B), OA (0.33 mM, C), OA (0.66mM, D) or OA (1 mM, E) in serum free medium. After 24 hr of treatment, cells were washed and fixed in formalin for oil red O (ORO) staining (red) and were observed under phase-contrast microscopy. Haematoxylin was used to counterstain nuclei (blue). (Right) ORO staining was extracted in isopropanol and absorbance was measured at 500nM. *p<0.05 (n=4 wells per treatment)

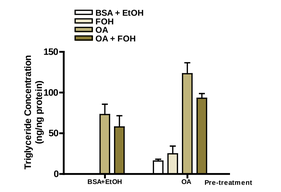

Figure 4: Farnesol co-treatment suppresses oleic acid (OA)-induced triglyceride (TG) accumulation in HepaRG cells. Differentiated HepaRG cells were pretreated with vehicle control (BSA+ethanol) or OA (0.66mM) in serum-free medium. After 24 hr, cells were treated with vehicle (BSA+ethanol), OA (0.66μM), farnesol (100μM), or OA and farnesol in combination. After 48 hr of treatment, cells were lysed and cellular TG levels were measured. Each bar represents the mean TG concentration (normalized to cellular protein) ± S.E.M of 3 independent experiments.

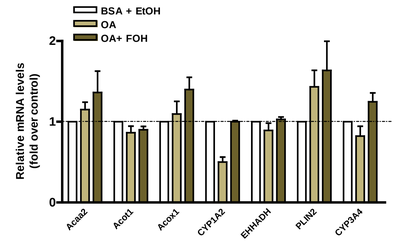

Figure 5: Effect of oleic acid and farnesol co-treatment on the expression of genes involved in fatty acid β-oxidation in HepaRG cells. Differentiated HepaRG cells were pretreated with either vehicle (BSA+ethanol) or OA (0.66mM) in serum-free media. After 24 hr, OA-pretreated cells were treated with OA (0.66μM) alone or in combination with farnesol (100μM). BSA-pretreated cells were treated with vehicle (BSA+ethanol). After 48 hr of treatment, cells were harvested and mRNA levels were measured. Each bar represents the mean mRNA level ± range relative to control (n=2 independent experiments)..

CONCLUSIONS

- Treatment of HepaRG cells with prototypical activators for CAR (CITCO), FXR (GW4064 and CDCA), or PPARα (GW764) increased expression of respective target genes, demonstrating that the CAR, FXR, and PPARα pathways are intact in HepaRG cells.
- Treatment with farnesol increased CYP2B6 mRNA levels, most likely through the activation of CAR. However, farnesol treatment did not increase SHP or PLIN2 expression, suggesting that farnesol has little effect on FXR or PPARα activity in HepaRG cells under standard culture conditions.
- Treatment with oleic acid produced a concentration-dependent increase in intracellular lipid levels, as observed by an increase in oil red O staining.
- Oleic acid-treated HepaRG cells also had higher intracellular TG levels compared to control. Co-treatment with farnesol lowered the OA-induced TG levels by ~25%. This suggests that farnesol may suppress fatty acid-mediated increases in intracellular TG levels.
- Treatment with oleic acid changed the expression of genes involved in hepatic fatty acid β-oxidation. CYP1A2 expression was decreased by OA treatment, and farnesol co-treatment restored the mRNA level back to control. OA treatment produced small increases in Acaa2, Acox1, and PLIN2 mRNA levels, which were further increased by farnesol treatment. These data suggest that farnesol can regulate the expression of genes involved in hepatic β-oxidation in HepaRG cells that are cultured under steatogenic conditions.

REFERENCES

1. Ahmed MH, Barakat S, Almobarak AO. (2012). J Obes, 483135.
2. Dam-Larsen S, Becker U, Franzmann MB, Larsen K, Christoffersen P, Bendtsen F. (2009). Scand J Gastroenterol, 44, 1236–43.
3. Su X and Abumrad NA. (2009). Trends Endocrinol Metab, 20, 72–7.
4. Duncan RE and Archer MC. (2008). Lipids, 43, 619–27.
5. Takahashi N, Kawada T, Goto T, Yamamoto T, Taimatsu A, Matsui N, Kimura K, Saito M, Hosokawa M, Miyashita K, Fushiki T. (2002). FEBS Lett, 514, 315–22.
6. Bostedor RG, Karkas JD, Arison BH, Bansal VS, Vaidya S, Germershausen JI, Kurtz MM, Bergstrom JD. (1997). J Biol Chem, 272, 9197–203.
7. Hiyoshi H. (2002). J Lipid Res, 44, 128–135.
8. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C. (2002). Proc Natl Acad Sci USA, 99,15655–15660.

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