Research Article

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Two non-psychoactive cannabinoids reduce intracellular lipid levels and inhibit hepatosteatosis

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Background & Aims: Obesity and associated metabolic syndrome have quickly become a pandemic and a major detriment to global human health. The presence of non-alcoholic fatty liver disease (NAFLD; hepatosteatosis) in obesity has been linked to the worsening of the metabolic syndrome, including the development of insulin resistance and cardiovascular disease. Currently, there are few options to treat NAFLD, including life style changes and insulin sensitizers. Recent evidence suggests that the cannabinoids Δ^9-tetrahydrocannabivarin (THCV) and cannabidiol (CBD) improve insulin sensitivity; we aimed at studying their effects on lipid levels.

Methods: The effects of THCV and CBD on lipid levels were examined in a variety of *in vitro* and *in vivo* systems, with special emphasis on models of hepatosteatosis. Transcriptional, post-translational and metabolomic changes were assayed.

Results: THCV and CBD directly reduce accumulated lipid levels *in vitro* in a hepatosteatosis model and adipocytes. Nuclear magnetic resonance- (NMR) based metabolomics confirmed these results and further identified specific metabolic changes in THCV and CBD-treated hepatocytes. Treatment also induced post-translational changes in a variety of proteins such as CREB, PRAS40, AMPKa2 and several STATs indicating increased lipid metabolism and, possibly, mitochondrial activity. These results are supported by *in vivo* data from zebrafish and obese mice indicating that these cannabinoids are able to increase yolk lipid mobilization and inhibit the development of hepatosteatosis respectively.

Conclusions: Our results suggest that THCV and CBD might be used as new therapeutic agents for the pharmacological treatment of obesity- and metabolic syndrome-related NAFLD/hepatosteatosis.

Introduction

The global number of overweight individuals almost tripled between 2008 and 2013, resulting in several million deaths annually [1]. Excessive weight, along with hypertriglyceridemia, hypertension and high fasting glucose levels are all hallmarks of the metabolic syndrome, which manifests itself in the liver as non-alcoholic fatty liver disease (NAFLD), characterized by hepatosteatosis resulting from elevated fatty acid influx as well as *de novo* lipid production [2]. Hepatosteatosis greatly impedes the ability of the liver to regulate total body energy homeostasis by impacting negatively on lipid and glucose metabolism. Although some contradictory data exist, it is now generally believed that NAFLD contributes to the metabolic syndrome, insulin resistance/type 2 diabetes mellitus and cardiovascular disease and not *vice versa* [3].

The endocannabinoid pathway encompasses signalling lipids (the two major endocannabinoids, anandamide [AEA] and 2-arachidonoylglycerol [2-AG]), G-protein-coupled receptors (the cannabinoid CB1 and CB2 receptors), and proteins regulating receptor activity and endocannabinoid levels. It is a key regulator of energy homeostasis, subsequently participating in the development of the metabolic syndrome [4]. Endocannabinoids are the endogenous ligands for CB1, which is the target for the psychoactive component of the *Cannabis* plant, Δ^9-tetrahydrocannabinol (THC). The liver expresses CB1 receptors and produces endocannabinoids, which regulate hepatic lipid metabolism and are involved in the development of both alcoholic and NAFLD [5,6]. Indeed, liver specific knockout of CB1, while not protecting from obesity, renders mice resistant to the development of hepatosteatosis and whole body insulin resistance [7].

Keywords: Fatty acid metabolism; Triglycerides; Cannabinoids; Endocannabinoids; Liver; Adipocytes; Obesity.
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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NMR, nuclear magnetic resonance; THCV, Δ9-tetrahydrocannabivarin; CBD, cannabidiol; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB1/2, Cannabinoid receptor 1/2; THC, Δ^9-tetra-hydrocannabinol; HHL-5, human hepatocyte line-5; OA, oleic acid; ECS, endocannabinoid system; TG, triglyceride; TRPV1, transient receptor potential vanilloid type-1; Cho, Choline; PC, phosphocholine; FA, fatty acid; GSH, glutathione; ATP, adenine triphosphate; NAD, nicotinamide adenine dinucleotide; FChol, free cholesterol; GK, Glycerol kinase; AMPK, 5′-AMP-activated protein kinase; OPLS-DA, Orthogonal Projection to Latent Structure Discriminant Analysis.

Cannabis has been used therapeutically for thousands of years, and although famous for THC, this is just one of dozens of cannabinoids produced by the plant. Δ^9-tetrahydrocannabivarin (THCV) and cannabidiol (CBD) are two non-psychoactive components of the *Cannabis* plant. While only the former compound binds potently to CB1 and CB2 receptors, both THCV and CBD can counteract the central actions of THC via different mechanisms, whereas several non-cannabinoid receptor targets have been identified, particularly for CBD [8]. Additionally, both compounds activate and desensitize the transient receptor potential vanilloid type-1 (TRPV1) channel [9], an emerging target in obesity and related cardiometabolic risk factors [10]. Importantly, CBD inhibits weight gain in rats on high-fat diets and the development of alcohol-induced hepatosteatosis in mice [11,12], whereas THCV improves insulin sensitivity and decreases triglyceride (TG) accumulation within the livers of obese mice [13]. THCV was recently shown to reduce insulin resistance and hypertension in a small double blind placebo-controlled clinical trial in type 2 diabetes patients with dyslipidemia [14]. These data indicate that both compounds are able to potentially elicit positive metabolic effects under conditions of an unbalanced diet. However, to date, little is known about the molecular mechanisms through which they modulate lipid homeostasis directly within cells.

We show here that both THCV and CBD reduce intracellular lipid levels *in vitro* in a model of hepatosteatosis and adipocytes, mobilize zebrafish embryo lipid yolk stores and inhibit hepatosteatosis in obese mice. qPCR, Western blot-based screening and nuclear magnetic resonance (NMR)-based metabolomic analysis identified cannabinoid-induced changes that may point to mechanisms of action for the lipid lowering effects of these compounds.

Materials and methods

Cell culture

Human hepatocyte line 5 (HHL-5) cells were cultured in standard growth media (DMEM 10% FBS, NEAA and Pen.Strep [all Gibco]). 3T3-L1 cells were cultured in growth media (DMEM, 10% FBS and Pen.Strep [all Gibco]) and differentiated 2 days post confluent in differentiation media (DMEM [Gibco], 10% FBS [Lonza], 1 μg/ml insulin [Sigma], 250 nM Dexamethasone [Sigma], 500 μM MIX [Sigma] and Pen.Strep [Gibco]) for 2 days, followed by incubation in insulin media (DMEM [Gibco], 10% FBS (Gibco), 1 μg/ml insulin [Sigma] and Pen.Strep [Gibco]), for 2 days. Cells were then cultured in growth media until mature. Cells were transfected with predesigned siRNAs (Life Technologies) utilizing Lipofectamine RNAi (Life Technologies) according to the manufacturer's instructions.

Triglyceride level analysis in cells

HHL-5 cells seeded in 96–well plates at 90% confluence and treated with 100 μM oleic acid (OA) or DMSO as indicated in the absence or presence of THCV and/or CBD [dissolved in DMSO; GW Pharmaceuticals (Salisbury, Wiltshire, UK)] at the indicated concentrations and times. 3T3-L1 cells were plated in 100 mm plates and differentiated as above to obtain mature adipocytes, re-plated into 48-well plates and then treated with the indicated concentrations of THCV or CBD for approximately 3 days. All cells then stained with AdipoRed (Lonza) and individual well fluorescence was measured in a 3 × 3 (HHL-5) or 5 × 5 (3T3-L1) grid with a Genios Pro Plate reader (Tecan) according to the manufacturer's instructions and data expressed as average and standard deviation.

Quantitative PCR and targeted transcriptome and proteome analysis

RNA was isolated from cells using Trizol (Invitrogen), Dnase I treated (Ambion) and reverse transcribed with SuperScript III RT reaction kit (Invitrogen) according to the manufacturers' instructions. 10 or 20 ng of RNA was then used for qPCR analysis in 10 or 20 μl reactions using IQ SybrGreen Supermix on a CFX 384 or IQ5 thermal cycler(BioRad) respectively. Data is expressed as relative mRNA levels with standard errors of the mean of triplicate reactions, as determined by CFX Manager software (BioRad). Targeted transcriptome and proteome analysis was performed using the Fatty Acid Metabolism RT^2 Profiler PCR Array (Qiagen) and Human Phospho-Kinase Array (R&D Systems) according to the manufacturers' instructions.

In vivo studies

Zebrafish (*Danio rerio*) embryos and larvae were obtained from a stable laboratory strain and raised at 28.5 °C on a 14L:10D photoperiod. Embryos were raised in filtered egg water and after hatching healthy larvae were staged, placed in 24-well plates at a density of 10 larvae/ml/well and exposed to drug solutions. Cannabinoids dissolved in DMSO were diluted with filtered egg water to 5 μM (DMSO 0.1%) and refreshed daily. Larvae were stained overnight with 0.005% AdipoRed (Lonza) permitting the staining of yolk without toxicity, anesthetized with tricaine (Sigma), mounted with 3% methyl cellulose (Sigma), photographed with a Tri-Red filter on a Leica DMI 6000B microscope and signal intensity analyzed with ImageJ 1,44p (NIH, USA).

Female *ob/ob* mice were treated by oral gavage with THCV (12.5 mg/kg) and CBD (3 mg/kg) for 4 weeks, after which livers were extracted and TG levels measured as per [13]. For details see Supplementary Materials and methods.

Cell extraction and sample preparation, NMR spectra acquisition and multivariate statistical analysis

Polar and lipophilic cell extracts were collected and extracts prepared as described previously [15]. NMR spectra of polar and apolar extracts were recorded at 600.13 MHz on a Bruker Avance-600 spectrometer equipped with a TCI CryoProbe™ fitted with a gradient along the Z-axis, at a probe temperature of 300 K. The resulting high resolution ^{1}H-NMR (1D) spectra were subjected to multivariate statistical analysis to identify trends and clusters [16]. For details see Supplementary Materials and methods.

Results

THCV and CBD dose- and time-dependently reduce lipid levels in an in vitro model of hepatosteatosis

We investigated the potential of THCV and CBD to directly modulate lipid metabolism within the liver HHL-5 (Human Hepatocyte Line 5) cells, which express components of the endocannabinoid system (ECS) (Supplementary Table 1), utilizing an OA-induced model of hepatosteatosis. After 24 h or 48 h of OA exposure HHL-5 cells greatly increased intracellular lipid levels, which was dose-dependently inhibited by co-incubation with THCV or CBD (Fig. 1). HHL-5 cells were next treated with OA for a total of 3 days and with 10 μM of THCV or CBD for either the full 3 days, the final 1 or 2 days, or only the first 2 days (Fig. 2A). THCV and CBD were similarly effective regardless of whether they were present for the entire time or only the final 1 or 2 days; however, their addition for only the initial 2 days had no effect on OA-mediated TG induction (Fig. 2A). These data suggest that THCV and CBD do not inhibit the upregulation of lipid production, but increase lipid clearance. To test this we treated HHL-5 cells with OA for 24 h and then with THCV or CBD at various time points before assessing TG levels. Upon removal of OA, untreated cells significantly decreased intracellular TG levels on their own after 24 h and the addition of THCV or CBD after OA removal resulted in further decreases (Fig. 2B). By 48 h after OA withdrawal, THCV- and CBD-treated cells showed TG levels similar to those of OA-untreated cells, by 8 days all samples had TG levels similar to OA-untreated cells. Assaying cells at earlier time points (4, 8, 12, 24, and 48 h) after cannabinoid treatment, either alone or concomitant with OA after an initial 24 h OA treatment, revealed that cells decreased intracellular TG levels over time after the initial