Biochemical Pharmacology 100 (2016) 86–97

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Fenretinide mediated retinoic acid receptor signalling and inhibition of ceramide biosynthesis regulates adipogenesis, lipid accumulation, mitochondrial function and nutrient stress signalling in adipocytes and adipose tissue

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ARTICLE INFO

Article history:
Received 19 August 2015
Accepted 16 November 2015
Available online 22 November 2015

Chemical compounds studied in this article:
Fenretinide (PubChem CID: 5288209)
4-oxo-*N*-(4-Hydroxyphenyl)retinamide (PubChem CID: 11258242)
Retinoic acid (PubChem CID: 444795)
Rosiglitazone (PubChem CID: 77999)

Keywords:
Fenretinide
Retinoic acid
Adipocytes
Metabolomics
Dihydroceramide
Lipidomics

ABSTRACT

Fenretinide (FEN) is a synthetic retinoid that inhibits obesity and insulin resistance in high-fat diet (HFD)-fed mice and completely prevents 3T3-L1 pre-adipocyte differentiation. The aim of this study was to determine the mechanism(s) of FEN action in 3T3-L1 adipocytes and in mice. We used the 3T3-L1 model of adipogenesis, fully differentiated 3T3-L1 adipocytes and adipose tissue from HFD-induced obese mice to investigate the mechanisms of FEN action. We measured expression of adipogenic and retinoid genes by qPCR and activation of nutrient-signalling pathways by western blotting. Global lipid and metabolite analysis was performed and specific ceramide lipid species measured by liquid chromatography-mass spectrometry. We provide direct evidence that FEN inhibits 3T3-L1 adipogenesis *via* RA-receptor (RAR)-dependent signaling. However, RARα antagonism did not prevent FEN-induced decreases in lipid levels in mature 3T3-L1 adipocytes, suggesting an RAR-independent mechanism. Lipidomics analysis revealed that FEN increased dihydroceramide lipid species 5- to 16-fold in adipocytes, indicating an inhibition of the final step of ceramide biosynthesis. A similar blockade in adipose tissue from FEN-treated obese mice was associated with a complete normalisation of impaired mitochondrial β-oxidation and tricarboxylic acid cycle flux. The FEN catabolite, 4-oxo-*N*-(4-hydroxyphenyl)retinamide (4-OXO), also decreased lipid accumulation without affecting adipogenesis. FEN and 4-OXO (but not RA) treatment additionally led to the activation of p38-MAPK, peIF2α and autophagy markers in adipocytes. Overall our data reveals FEN utilises both RAR-dependent and -independent pathways to regulate adipocyte biology, both of which may be required for FEN to prevent obesity and insulin resistance *in vivo*.

1. Introduction

Retinol (vitamin A) and the retinoid metabolism pathway play an important role in body mass regulation and adipocyte biology [1–6]. Targeting retinoid homeostasis may therefore offer a therapeutic approach for obesity and type-2 diabetes. Vitamin A is a lipid soluble molecule which undergoes multiple steps of metabolism through a complex pathway of enzymes and transport proteins [7]. All-*trans*-retinoic acid (RA), which is the most active metabolite of retinol, has long been known to inhibit adipogenesis through the prevention of C/EBPβ mediated transcription [1,8], More recently, RA has been shown to improve obesity and glucose homeostasis *in vivo* [4]. However, with prolonged exposure, naturally derived retinoid compounds such as RA and retinyl-acetate can lead to liver toxicity, which restricts their potential use as therapeutic agents [9].

Fenretinide (FEN), otherwise known as *N*-(4-hydroxyphenyl) retinamide or 4-HPR, is a structural derivative of RA with reduced

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http://dx.doi.org/10.1016/j.bcp.2015.11.017

toxicological profile [10,11]. FEN treatment leads to increased renal clearance and thus decreased serum levels of the retinol transport protein, serum retinol binding protein (gene name *Rbp4*) in both humans and mice [12–16]. This decrease in circulating RBP4 levels had been proposed to be the mechanism that FEN treatment led to prevention of insulin resistance associated with high-fat diet (HFD) induced obesity [15]. However, FEN has also been shown to reduce obesity and hyperleptinaemia in mice lacking RBP4, implying that the anti-obesity effects of FEN are most likely to be independent of its ability to decrease circulating RBP4 levels [16].

Thus, the beneficial effects of FEN appear to be through several different mechanisms including alterations to retinoid homeostasis in multiple tissues [6], increased hepatic lipid oxidation [17] and inhibition of ceramide biosynthesis leading to an increase in dihydroceramide in muscle and liver [18]. Studies in cancer cells have found that both FEN and dihydroceramide treatment are associated with the activation of cellular stress responses and autophagy induction [19–21]. Autophagy plays a crucial role in cellular homeostasis through the degradation and recycling of organelles such as mitochondria or ER and the regulation of intracellular lipid stores [22,23]. Since defective autophagy may also underlie impaired insulin sensitivity in obesity and upregulating autophagy may be a useful strategy to combat insulin resistance [24–26], it highlights the importance to further characterise the biological effects of FEN.

In addition to the beneficial effect of FEN *in vivo*, we recently demonstrated that FEN (similarly to RA) is able to inhibit 3T3-L1 adipocyte differentiation by blocking transcription of C/EBPα and PPARγ, master regulators that synergistically coordinate adipogenesis and adipocyte biology and simultaneously increasing retinoid gene expression [6]. Interestingly, rosiglitazone (ROSI), a thiazolidinedione and PPARγ agonist commonly used to stimulate adipogenesis, suppressed gene expression of all the retinoid metabolism markers examined in differentiating 3T3-L1 cells [6]. FEN also decreased lipid accumulation in fully differentiated 3T3-L1 adipocytes [6], but RA treatment did not [27,28]. Thus, FEN and RA appear to have divergent biological effects which may be due to unique activation of retinoic acid receptor (RAR)-dependent and -independent pathways. Since, it is unknown which pathway FEN requires for its biological effects in adipocytes we aimed to determine the mechanism(s) of FEN action in the 3T3-L1 model of pre-adipocyte differentiation and fully differentiated adipocytes. In addition, we have translated some of our new findings to adipose tissue of HFD-induced obese mice.

2. Methods

2.1. Cell culture

3T3-L1 pre-adipocytes were maintained and differentiated for 8 days (or 16 days where stated) as previously described [6]. C3H10T1/2 cells were similarly treated however penicillin/streptomycin was omitted from the media. DMSO was used as vehicle control (VEH) and to dissolve all experimental compounds. FEN (Cilag AG, Schaffhausen, Switzerland), RA (Sigma–Aldrich, UK) and ROSI (Cayman Chemical, MI, USA) were used at 1 μM, 4-OXO (Santa Cruz, TX, USA) at 0.5 μM and ER50891 (Tocris Bioscience, Bristol, UK) at 10 μM and added at day 0 (or day 8 where indicated). Cells were stained for neutral lipids with Oil Red O, images taken and then the stain was eluted and quantified at 520 nm.

2.2. Lipolysis assay

Basal glycerol levels were measured in media collected at day 16 of differentiation from 3T3-L1 cultures. The Triglyceride Liquid assay (Sentinel Diagnostics #17628) was used following the supplied protocol.

2.3. Gene expression and protein analysis

RNA isolation, cDNA synthesis and qPCR were performed at day 8 of differentiation (or as stated in the figure legends) as previously described [6]. Control reactions for contaminating DNA were performed routinely and relative expression calculated using the Pfaffl method [29]. The geometric mean of three stable reference genes (*Nono*, *Ywhaz* and *Actb* or as stated in the figure legend) were obtained from five commonly used sequences and used for normalisation. Primer sequences available on request, some of which were obtained from PrimerBank [30].

SDS-PAGE was performed and transferred to nitrocellulose membranes as described previously [31]. Antibodies against p-eIF2α (#9721), eIF2 α (#5324S), p-p38 MAPK (#9211), p38 MAPK (#8690S), Beclin1 (#3495), LC3B (#3868), p-Akt Ser473 (#9271) were from Cell Signalling, SH-PTP2 (sc-280) and Akt1/2/3 (sc-8312) from Santa Cruz. All antibodies were detected with goat anti-rabbit HRP secondary antibody (#28177) from Anaspec. Proteins were visualized using enhanced chemiluminescence (ECL) and quantified by densitometry scanning using the Fusion imaging system and Bio-1D software (Peqlab).

2.4. Global lipidomics analysis of adipocytes

Extraction of 3T3-L1 adipocyte lipids was performed according to the method described by Folch et al. [32]. The lipids were analysed by liquid chromatography–mass spectrometry (LC–MS) using a Thermo Orbitrap Exactive mass spectrometer (Thermo Scientific, Hemel Hempstead, UK), equipped with a heated electrospray ionization (HESI) probe and coupled to a Thermo Accela 1250 UHPLC system. All samples were analysed in both positive and negative ion mode over the mass to charge (m/z) range 200–2000. The samples were injected on to a Thermo Hypersil Gold C18 column (2.1 mm × 100 mm, 1.9 μm). Mobile phase A consisted of water containing 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B consisted of 90:10 isopropanol/acetonitrile containing 10 mM ammonium formate and 0.1% (v/v) formic acid. The initial conditions for analysis were 65%A/35%B. The percentage of mobile phase B was increased to 100% over 10 minutes and held for 7 min before re-equilibration with the starting conditions for 4 min. All solvents were LC–MS grade (Fisher Scientific, Loughborough, UK). The raw LC–MS data were processed with Progenesis CoMet v2.0 software (Non-linear Dynamics, Newcastle, UK) and searched against LIPID MAPS (www.lipidmaps.org) for identification.

2.5. Animals

Male C57BL/6 mice were randomised by body weight at three months of age and fed CHOW, HFD or FEN-HFD for 20 weeks. Analysed tissues were collected during previously performed experiments as described in [6], whereby FEN-HFD prevented obesity and factors associated with insulin resistance. Perigonadal white adipose tissue (PG-WAT) from *ad libitum* fed mice was rapidly dissected, frozen in liquid nitrogen, and stored at −80 °C. Animal procedures were approved by the University of Aberdeen Ethics Review Board and performed under license (PPL60/3951) approved by the UK Home Office.

2.6. Quantitative analysis of ceramides and dihydroceramides in adipose tissue

Lipids were extracted from murine adipose tissue according to the method of Bligh and Dyer [33]. The ceramides and