Toxicology Letters 262 (2016) 92–99

Contents lists available at ScienceDirect

Toxicology Letters

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

Parabens inhibit fatty acid amide hydrolase: A potential role in paraben-enhanced 3T3-L1 adipocyte differentiation

Sean D. Kodani[a], Haley B. Overby[b], Christophe Morisseau[a], Jiangang Chen[b,c], Ling Zhao[b], Bruce D. Hammock[a,*]

[a] Department of Entomology and Nematology, and UC Davis Comprehensive Cancer Center, University of California, Davis, CA 95616, USA
[b] Department of Nutrition, The University of Tennessee, Knoxville, TN 37996, USA
[c] Department of Public Health, The University of Tennessee, Knoxville, TN 37996, USA

HIGHLIGHTS

- Parabens inhibit the endocannabinoid enzyme fatty acid amide hydrolase (FAAH).
- Paraben inhibition has time-independent, mixed-type kinetics.
- Benzylparaben, the most potent paraben, inhibits FAAH with sub-micromolar potency.
- Endocannabinoids may mediate paraben-enhanced adipogenesis but not through CB_1 activation.

ARTICLE INFO

Article history:
Received 18 May 2016
Received in revised form 2 August 2016
Accepted 18 September 2016
Available online 19 September 2016

Keywords:
Parabens
Benzylparaben
Fatty acid amide hydrolase
Adipocyte

ABSTRACT

Parabens are a class of small molecules that are regularly used as preservatives in a variety of personal care products. Several parabens, including butylparaben and benzylparaben, have been found to interfere with endocrine signaling and to stimulate adipocyte differentiation. We hypothesized these biological effects could be due to interference with the endocannabinoid system and identified fatty acid amide hydrolase (FAAH) as the direct molecular target of parabens. FAAH inhibition by parabens yields mixed-type and time-independent kinetics. Additionally, structure activity relationships indicate FAAH inhibition is selective for the paraben class of compounds and the more hydrophobic parabens have higher potency. Parabens enhanced 3T3-L1 adipocyte differentiation in a dose dependent fashion, different from two other FAAH inhibitors URB597 and PF622. Moreover, parabens, URB597 and PF622 all failed to enhance AEA-induced differentiation. Furthermore, rimonabant, a cannabinoid receptor 1 (CB_1)-selective antagonist, did not attenuate paraben-induced adipocyte differentiation. Thus, adipogenesis mediated by parabens likely occurs through modulation of endocannabinoids, but cell differentiation is independent of direct activation of CB_1 by endocannabinoids.

1. Introduction

The biologic activity of many high production volume small molecules in consumer care products is poorly understood but is of high concern due to their daily use. Compared to environmental toxicants where individuals are unintentionally exposed to low doses of chemicals, many of these chemicals are voluntarily applied at high doses directly to the skin. Of these consumer care product chemicals, parabens are a class of small molecules commonly added to pharmaceuticals, cosmetics and food products for their antimicrobial activity (Bledzka et al., 2014). Structurally, they are esters of *p*-hydroxybenzoic acid and most commonly

Abbreviations: AADAC, arylacetamide deacetylase; AEA, arachidonoyl ethanolamide; BnP, benzylparaben; BuP, butylparaben; CB_1R, cannabinoid receptor 1; CB_2R, cannabinoid receptor 2; CMNA, cyano(6-methoxynaphthalen-2-yl)methyl acetate; EC, endocannabinoid; FAAH, fatty acid amide hydrolase; GR, glucocorticoid receptor; hCE1, carboxylesterase 1; hCE2, carboxylesterase 2; OMP, N-(6-methoxypyridin-3-yl) octanamide; PF-622, N-phenyl-4-(quinolin-2-ylmethyl)piperazine-1-carboxamide; PPARγ, peroxisome proliferator-activated receptor γ; URB597, 3′-carbamoyl-[1,1′-biphenyl]-3-yl cyclohexylcarbamate.

* Corresponding author at: Department of Entomology and Nematology, University of California, Davis, Davis, CA 95616, USA.
E-mail address: bdhammock@ucdavis.edu (B.D. Hammock).

http://dx.doi.org/10.1016/j.toxlet.2016.09.011
0378-4274/

include methylparaben, propylparaben and butylparaben. They are not acutely toxic with oral LD_{50} values > 1 g/kg (Soni et al., 2005). Furthermore, methylparaben and propylparaben are on the Food and Drug Administration's list of chemicals "generally recognized as safe" (FDA, 1973). However, despite the low acute toxicity there has been significant concern over whether they act chronically as endocrine disrupting chemicals with studies providing evidence for (Chen et al., 2007) and against (Hoberman et al., 2008) such arguments.

In addition to their possible endocrine disrupting effects as sex hormone mimics, parabens have been reported to stimulate adipocyte differentiation (Taxvig et al., 2012; Hu et al., 2013). Adipocytes are formed early in life and the number of adipocytes does not generally increase in lean adults. However, it is possible for precursor cells to differentiate into adipocytes in adults when the demand for energy storage increases (Wang et al., 2014). Paraben-enhanced differentiation would supplement this process and increase the prevalence of obesity within exposed populations. Adipogenic effects are suggested to be mediated through the peroxisome proliferator-activated receptor gamma (PPARγ) and the glucocorticoid receptor (GR). Antagonism of either PPARγ or the GR using small molecules partially reduced the paraben-enhanced differentiation (Hu et al., 2013). Butylparaben, but not benzylparaben, activate the PPARγ receptor and all parabens tested, including butylparaben and benzylparaben, activated the GR but do not compete with dexamethasone binding to the GR (Hu et al., 2013; Pereira-Fernandes et al., 2013). Thus, while PPARγ and GR may be involved in paraben-mediated differentiation, they are not the direct targets responsible for all of these effects.

In addition to PPARγ and the GR, evidence exists to implicate the endocannabinoid (EC) system in the regulation of adipogenesis and general energy homeostasis (Silvestri and Di Marzo, 2013). Signaling through the EC system primarily occurs through two lipid mediators, arachidonoyl ethanolamide (also known as anandamide, AEA) and 2-arachidonoylglycerol (2-AG) that bind to the cannabinoid receptors (CB_1 and CB_2) (Pertwee, 2015). Independent of food intake, blockage of the CB_1 receptor reduces lipogenesis (Vida et al., 2014). The CB_1 antagonist rimonabant was used in clinical trials for the treatment of obesity, but it was pulled from the market due to serious side effects including depression (Christopoulou and Kiortsis, 2011). Pharmacologic and genetic knockout of the CB_1 receptor seems to reduce or ablate glucocorticoid stimulated weight gain, indicating these effects occur downstream of the GR (Bowles et al., 2015). In addition to their action on the CB_1 receptor, endocannabinoids may target additional pathways to affect metabolism. For example, AEA has been shown to bind directly to PPARγ to mediate adipocyte differentiation (Bouaboula et al., 2005; Karaliota et al., 2009).

Given the role of the EC system on adipocyte differentiation, we hypothesized previously observed effects of parabens on adipocytes may be mediated through modulation of endocannabinoid signaling. To test this hypothesis, we directly tested the ability of parabens to inhibit fatty acid amide hydrolase (FAAH), the enzyme primarily responsible for regulating concentrations of AEA. In addition, the effects of parabens, other FAAH inhibitors, and their interaction with AEA on 3T3-L1 adipocyte differentiation were explored.

2. Materials and methods

2.1. Chemicals

Methylparaben, propylparaben, butylparaben, benzylparaben and 4-hydroxybenzoic acid were all purchased from Acros Chemicals. URB597 and PF622 were purchased from Cayman Chemical and heptylparaben was purchased from Sigma-Aldrich. Other paraben-like compounds described were synthesized as described in Supplementary material. N-(6-methoxypyridin-3-yl) octanamide (OMP) and cyano(6-methoxynaphthalen-2-yl)methyl acetate (CMNA) were synthesized as previously described (Shan and Hammock, 2001; Huang et al., 2007).

2.2. Preparation of enzyme extracts

The transgenic production of the FAAH enzyme and other esterases in baculovirus are previously described (Nishi et al., 2006; Huang et al., 2007). A crude preparation of enzyme was prepared by centrifuging cells at 1000 rpm, 15 min, 4 °C and re-suspending the pellet in 50 mM tris buffer (pH = 8.0) with 1 mM benzamidine and 1 mM EDTA. The solution was homogenized (3 × 15 s) and centrifuged (9000*g*, 20 min). The resulting pellet was re-suspended in 50 mM tris/HCl buffer (pH = 8.0) with 1 mM CHAPS and 10% glycerol and kept frozen at −80 °C until use. The amount of FAAH in the crude extract was estimated to be 5% by SDS-PAGE.

For measuring FAAH inhibition, rat and mouse microsomes were prepared from frozen brain tissue. Tissue was homogenized (3 × 15 s) in 20 mM phosphate buffer (pH = 7.4) with 5 mM EDTA and centrifuged (9,000*g*, 20 min). The soluble fraction (S9) was collected and centrifuged again (100,000*g*, 1 h). The pellet containing microsomes was resuspended in 10 mM phosphate buffer with 2.5 mM EDTA and 20% glycerol and kept frozen at −80 °C until use. Treatment with 50 nM of the potent FAAH inhibitor URB597 reduced OMP hydrolysis to less than 20%, indicating most if not all of the activity measured is from FAAH.

2.3. Fluorimetric enzyme assays

FAAH activity was measured in 0.1 M sodium phosphate buffer at pH = 8 with 0.1-0.2 mg/mL of bovine serum albumin (BSA) using OMP as the substrate (Huang et al., 2007). Formation of the fluorescent methoxypyridine product was measured kinetically at $\lambda_{excitation}$ = 303 nm and $\lambda_{emission}$ = 394 nm while reaction solutions were kept at 37 °C. To determine IC_{50} values, final reaction solutions contained approximately 0.9 μg of crude FAAH extract, $[S]_{final}$ = 50 μM and inhibitor in no more than 2% DMSO solution. Esterase activity (hCE1, hCE2 and AADAC) was measured under the same conditions using CMNA as the substrate and measuring the liberation of 6-methoxynaphthaldehyde at $\lambda_{excitation}$ = 330 nm and $\lambda_{emission}$ = 465 nm (Morisseau et al., 2009). All experiments were run in either duplicate or triplicate and values reported as average ± SD represent at least 3 independent experiments.

2.4. Cell culture, induction of adipocyte differentiation and chemical treatments

Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone) in a 5% CO_2, 37 °C environment until they reach confluence. To study the potentiating effects of parabens and other chemicals on adipocyte differentiation, the standard differentiation protocol was modified using a weaker GR agonist cortisone as previously described (Hu et al., 2013). Briefly, on the day of reaching confluence (designated as day 0), cells were treated with DMEM containing 10% fetal bovine serum (FBS, Atlas Biologicals), 0.5 mM methylisobutylxanthine (MIX), 170 nM insulin and 5 μM cortisone for 3 days. The cells were then grown in maintenance DMEM containing 10% FBS and 170 nM insulin for additional two days until day 5 followed by in DMEM containing 10% FBS until day 7.

Parabens and other chemicals were added in the differentiation media of 3T3-L1. Parabens and other chemicals were applied at