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Effect of endocannabinoid oleamide on rat and human liver cytochrome P450 enzymes in in vitro and in vivo models

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List of abbreviations:

AhR – aryl hydrocarbon receptor; CAR – constitutive androstane receptor; CITCO – (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime); CYPs – cytochromes P450; ECS – endocannabinoid system; FAAH – fatty acid amide hydrolase; LBD – ligand binding domain; PXR – pregnane X receptor; RLMs – rat liver microsomes

Abstract

The endocannabinoid system is important for many physiological and pathological processes, but its role in the regulation of liver cytochromes P450 (CYPs) is still unknown. We studied the influence of the endocannabinoid oleamide on rat and human liver CYPs. Oleamide was administered i.p. to rats at doses of 0.1, 1 and 10 mg/kg/day for 7 days. The content and activity of key CYPs was evaluated in rat liver microsomes. Moreover, its interactions with nuclear receptors regulating CYP genes and serum levels of their ligands (prolactin, corticosterone, and free triiodothyronine) were tested in vitro CYP inhibition assays. Decreased protein levels and metabolic activities of CYP1A2, CYP2B, and CYP2C11 along with a drop in metabolic activity of CYP2D2 were observed in animals treated with oleamide (10 mg/kg/day). The activities of CYP2C6, CYP2A, and CYP3A and the levels of hormones were not altered. In vitro, oleamide exhibited a weak inhibition of rat CYP1A2, CYP2D2, and CYP2C6. The activities of rat CYP2A, CYP2B, CYP2C11, and CYP3A and human CYP1A2, CYP2B6, CYP2C9, and CYP3A4 were not altered. Oleamide did not interact with human pregnane X, constitutive androstane and aryl hydrocarbon receptors in reporter gene experiments and did not regulate their target CYP genes in primary human hepatocytes. Our results indicate that oleamide caused the downregulation of some rat liver CYPs, and hormones are not mediators of this effect. In vitro oleamide inhibits mainly rat CYP2C6 and it is neither an agonist nor antagonist of major human nuclear receptors involved in the regulation of xenobiotic metabolism.

1. Introduction

The endocannabinoid system (ECS) is an essential regulator of many physiological processes, such as memory, brain plasticity, thermogenesis, nociception, energy balance, digestion, motility and fertility (Aizpurua-Olaizola et al., 2017). Therefore, it is not surprising that changes in ECS activity are involved in many pathophysiological processes in the central nervous system, as well as in the peripheral tissues (Pertwee, 2015). The ECS is a signalling system which consists of cannabinoid receptors CB_1 and CB_2, their endogenous ligands (endocannabinoids), and associated proteins for their synthesis, transport, and degradation. Endocannabinoids are mainly metabolised by enzymatic hydrolysis via fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase, but some endocannabinoids can also be sensitive to oxidative metabolism by cyclooxygenase, lipoxygenase, and cytochrome P450 enzymes (CYPs) (Sugiura et al., 2002; Snider et al., 2010). N-arachidonoyl-ethanolamine (anandamide) and 2-arachidonoyl-glycerol are two of the best-studied endocannabinoids (Di Marzo et al., 1998). Apart from these two, oleamide (syn. cis-9,10-octadecenoamide, cis-9-octadecenamide, oleic acid amide) is another molecule of interest because of its role in sleep regulation (Krueger, 2003). It was first identified in the cerebrospinal fluid of sleep-deprived cats (Cravatt et al., 1995). Because of their similar structures, oleamide and anandamide evoke similar effects (Divito and Cascio, 2013) including sleep induction (Herrera-Solis et al., 2010), hypothermia, hypomotility, and analgesia (Smith et al., 1994; Fedorova et al., 2001). Oleamide is an agonist of CB_1 receptors (Leggett et al., 2004). Moreover, it can act as an alternate substrate for FAAH and thus increases the relative half-life and effects of anandamide (Fowler et al., 2001). Synthetically produced oleamide has been used in industry for a long time as a lubricant in the production of plastic materials (Garrido-Lopez et al., 2006), and as a thickener and emulsifier in cosmetics (Flick, 2012).

CYPs are the major enzymatic systems of drug metabolism. In particular, the subfamilies CYP3A, CYP2C, CYP2D, CYP2B, and CYP1A are essential for the biotransformation of xenobiotics in humans. It is known that many cannabinoids are not only substrates of CYPs,

but they can also change the activity of these enzymes. Nowadays therapeutically used phytocannabinoids (Δ^9-tetrahydrocannabinol, cannabidiol) and synthetic cannabinoids (dronabinol and nabilone) are known to modulate the activity of CYPs in in vitro and in vivo models (Zendulka et al., 2016). Their main indications in clinical practice include nausea and vomiting associated with cancer chemotherapy, pain, and spasticity (May and Glode, 2016; Keating, 2017). In addition, the ECS is a promising target for new therapeutic strategies for other diseases e.g. psychiatric disorders, obesity, diabetes, and osteoarthritis (Aizpurua-Olaizola et al., 2017). Thus, its possible role in the regulation of the activity of CYPs would be of great interest. Nevertheless, none of the endocannabinoids have been tested for their influence on liver CYPs yet.

We focused on oleamide as a candidate endocannabinoid for our study because of its known ability to activate the ECS by various mechanisms (CB_1 receptor and FAAH enzyme). Moreover, the wide use of oleamide in the plastics industry made it a possible pollutant and the knowledge of its effect on CYPs would be beneficial.

The aim of this work was to investigate the influence of the endocannabinoid oleamide on the activities of key rat and human CYPs involved in drug metabolism. For this purpose, in vitro CYP interaction assays and an in vivo rat model were used. To obtain additional data about the possible mechanism of oleamide's influence on CYPs, we also measured the serum concentrations of hormones known to be involved in the regulation of CYP metabolic activity, namely prolactin, corticosterone, and free triiodothyronine (T3). Finally, the interactions of oleamide with human nuclear receptors that regulate the expression of CYP genes, namely the Pregnane X Receptor (PXR), Aryl Hydrocarbon Receptor (AhR), and Constitutive Androstane Receptor (CAR) were also tested. The ability to induce their target CYPs genes was examined in primary human hepatocytes.

2. Material and methods

2.1. Chemicals

The following compounds were provided by Sigma-Aldrich (St. Louis, USA): oleamide (CAS No. 301-02-0), prednisone, testosterone, chlorpropamide, phenacetin, diclofenac, 4′-hydroxydiclofenac, acetaminophen, ibuprofen, dextromethorphan, dextrorphan, laudanosine, triethylamine, NADP, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, $MgCl_2$, EDTA, KH_2PO_4, Na_2HPO_4, sucrose, NH_4HCO_3, CH_3COOH, NH_3, KCl, bovine serum albumin, DMSO, α-naphthoflavone, ketoconazole, fluconazole, ticlopidine, rifampicin, CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) and 3-methylcholanthrene. Propylene glycol was obtained from Fagron (Olomouc, CZE). The metabolites of testosterone, namely 2β-, 2α-, 7α-, 6β-, 16α-, and 16β-hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI, USA). The primary and secondary antibodies, which were used for the western blotting analyses, are described in detail in Table 1. Chemicals and organic solvents for HPLC analysis were purchased from Lach-ner (Neratovice, CZE) and they were of an HPLC-gradient or LC-MS grade. Cell culture media, non-essential amino acids and fetal bovine serum were purchased from Thermo Fisher Scientific, Inc. (Waltham, USA).

2.2. Animals

The experiments were carried out on male Wistar albino rats (230 ± 20 g), which were purchased from the Masaryk University breeding facility (Brno, CZE). The rats were housed under standard laboratory conditions (22 ± 2 °C room temperature; 55 ± 5 % room humidity; 12/12 h light/dark cycle). Animals had free access to water and food. All experiments were performed in accordance with Czech act No. 246/1992, and with the approval of both the regional and national Czech Central Commission for Animal Welfare (MSMT-35 473/2012-30).

2.3. Experimental design

After 5 days of acclimatization, the rats were randomly divided into five experimental groups. All animals were treated i.p. for 7 consecutive days. Three groups (n=10/group) were administered with oleamide dissolved in propylene glycol at doses of 0.1, 1, and 10 mg/kg/day. The doses of oleamide used in our experiment were selected according to preclinical studies in which oleamide evoked various kinds of biological activities (Fedorova et al., 2001; Huitron-Resendiz et al., 2001). The control group was treated with the appropriate volume of the vehicle (propylene glycol 1 ml/kg, n=6). Because there was no data on the influence of propylene glycol on CYPs, a group of animals that were administered saline (1 ml/kg, n=10) was also used. All animals were sacrificed under ether anaesthesia by decapitation 24 h after the last drug/vehicle administration and the samples of liver tissue were drawn immediately without any liver perfusion and were frozen at -80 °C.

2.4. Preparation of rat liver microsomes and determination of total protein and CYP content

Rat liver microsomes (RLMs) were isolated from 3 g of liver tissue from each individual animal. Each liver tissue sample was homogenized in 20 mM Tris/KCl buffer (pH=7.4). RLMs were then isolated from the homogenate by differential ultracentrifugation (19,000 × g, 20 min and 2x 105,000 × g, 60 min) in 20 mM Tris/KCl buffer (pH=7.4) including washing with 0.15 M KCl. The final pellet was reconstituted with 0.25 M Tris/sucrose buffer (pH=7.4) to yield 1 ml of RLMs from each gram of isolated tissue and stored at -80 °C. The total protein content in the microsomal preparations was assessed according to the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin as a standard. The determination of total CYP content was assessed using a CO difference spectroscopy method according to Omura et al. (Omura and Sato, 1964). The results of total protein content and total CYP content are expressed as mg of protein per ml and nmol of CYP per ml of RLMs, respectively. RLMs were reconstituted in volume of Tris/sucrose buffer to resemble the original liver tissue content.

2.5. Determination of cytochrome P450 activity in rat liver microsomes

The assessment of the relative activity of CYP1A2, CYP2B, CYP2C, CYP2D2, and CYP3A enzymes were based on the rate of biotransformation of a specific marker in RLMs with an NADPH generating system according to the previously described method of Wojcikowski et al. (Wojcikowski et al., 2008), with a slight modification. The incubation mixture of the final 0.5 ml volume contained a phosphate buffer (50 mM; pH=7.4), EDTA (1.1 mM), NADP (1.2 mM), glucose-6-phosphate (4.4 mM), $MgCl_2$ (3.2 mM), glucose-6-phosphate dehydrogenase (1 U/ml), RLMs (1 mg/ml of total protein content), and a specific marker for the determination of the selected CYP enzymes activity. The reaction was stopped after incubation at 37 °C in a shaking water bath by the addition of 50 µl of methanol and cooling in ice. The concentrations of markers and their specific metabolites were measured with one of two HPLC systems: a Shimadzu LC-10 (Shimadzu, Tokyo, JPN) with a DAD detector (SPD-M10AVP) and a fluorescence detector (RF 10AXL) or with a Dionex UltiMate 3000RS LC (Thermo Fisher Scientific, Waltham, USA) with a DAD detector. Reaction rates exhibited a linear relationship to the incubation time and protein concentration under the above-mentioned conditions. The relative metabolic activities of all CYPs were studied by measuring the rates of CYP-specific reactions and were expressed as a molar concentration of the metabolite/min/mg of the total protein in RLMs. The specific metabolic activities were also calculated with respect to the total CYP content and are expressed as the molar concentration of the metabolite/min/nmol of total CYP content. The accuracy and precision of the HPLC methods were below 10% of RSD within the calibration range. The calibration range always included the concentrations of analytes in the measured samples.

2.5.1. Determination of CYP1A2 activity

The rate of phenacetin *O*-deethylation was used for CYP1A2 activity assessment. The substrate concentration was 300 µM and the incubation period was 20 min. After addition of the internal standard (chlorpropamide) to the incubation mixture, the analytes were extracted from the

microsomal suspension with diethyl ether (4 ml, 10 minutes of horizontal vortexing, 1400 rpm), the organic phase was evaporated, and the residue was dissolved in 250 µl of the mobile phase. The following gradient mode of separation was applied: 0:00 to 5:00 min – 90:10 (v/v 0.1% NH_4CH_3COO/acetonitrile, pH 4.6), from 5:00 to 6:00 min – 35:65, and from 6:00 to the end of the analysis at 8:00 min – 90:10. The separation of acetaminophen, phenacetin, and chlorpropamide was achieved using Kinetex 2.6 µm PFP 100A column (150 × 4.60 mm, Phenomenex, Torrance, USA) thermostated at 45 ±0.1 °C, at a flow rate of 1.2 ml/min, and UV detection at 245 nm.

2.5.2. Determination of CYP2A, CYP2B, CYP2C11, CYP3A activity

Testosterone 7α-, 16β-, 2α-, and 16α-, 2β-, and 6β-hydroxylations were used for the activity assessment of CYP2A, CYP2B, CYP2C11, and CYP3A, respectively. The substrate concentration was 400 µM, prednisone was used as the internal standard, and the incubation time was 15 min. The separation conditions for testosterone metabolite determination have been described in detail previously (Dovrtelova et al., 2015).

2.5.3. Determination of CYP2C6 activity

Diclofenac 4′-hydroxylation was used for measuring CYP2C6 activity, and the substrate concentration was 100 µM. Ibuprofen was used as the internal standard, and the incubation time was 20 min. The separation conditions for diclofenac and 4′-hydroxydiclofenac determination have been described in detail previously (Noskova et al., 2016).

2.5.4. Determination of CYP2D2 activity

Dextromethorphan *O*-demethylation was used for measuring CYP2D2 activity. The substrate concentration was 500 µM and the incubation time was 20 min. After addition of the internal standard (laudanosine) and Na_2CO_3 (250 µl, 0.5 M) to the incubation mixture, the analytes were extracted from the microsomal suspension with 4 ml of 1-butanol:hexane mixture (1:9). The organic phase was decanted and 300 µl of 0.01 M HCl were added. After 10 minutes of horizontal shaking at 1400 rpm, the samples were frozen. The organic layer was discarded and

a 5 µl portion of the aqueous phase was injected into the HPLC system. The separation of dextrorphan, dextromethorphan, and laudanosine was achieved in an isocratic separation mode with fluorescence detection according to the method of Zimova et al. (Zimova et al., 2000).

2.6. Western blotting

Western blot analyses were used to detect the protein expression of CYP2B1/2B2, CYP3A1, CYP1A2, and CYP2C11 in RLMs. Individual samples were mixed with a loading buffer (0.125 M Tris pH 6.8, 20% glycerol, 10% mercaptoethanol, 4% SDS, 0.004% bromphenol blue), boiled for 5 min at 95 °C and separated on a 12% SDS-PAGE gel. 10 µg of RLMs was loaded per lane. After the electrophoresis, proteins were electro-transferred (100 V, 75 min) onto polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, DEU), which were blocked with 5% non-fat milk in TBS-T (Tris-Buffered Saline with 0.08% Tween 20) at room temperature for 1 hour. The membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with the appropriate secondary antibodies for 1 hour at room temperature. The antibodies were dissolved according to Table 1 and the β-actin antibody was used as a loading control for normalization. Where necessary, the membranes were stripped in a stripping buffer (74.3:25:10:0.7 H_2O:1 M Tris (pH 7.4):20% SDS:β-mercaptoethanol), washed, and reblotted with another antibody. The intensities of the bands on the membranes were visualised using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Watford, GBR) and FUSION SL (Vilber Lourmat, DEU). The western blots were quantified using the software ImageJ (version 1.48, National Institutes of Health, Bethesda, USA). Statistical analyses were done using at least three repetitions of individual animals of each group.

2.7. CYP inhibition assay in vitro

RLMs from drug naïve control animals, which were pooled from 4 individuals, were used for a direct inhibition assay in vitro**.** The incubation mixtures were processed as described in Section 2.5., except the final volume was 1 ml and the total protein content in RLMs was

0.25 mg/ml. Each sample was prepared in triplicate. After 10 min of pre-incubation with a different concentration of oleamide (see below) at 37 °C in a shaking water bath, reactions were initiated by the addition of specific markers. The incubation times were 40 min (phenacetin and dextromethorphan), 20 min (testosterone), and 30 min (diclofenac).

First of all, pilot experiments were performed with single concentrations of the substrates phenacetin (300 μM), testosterone (100 μM), diclofenac (8 μM), or dextromethorphan (28 μM), and three concentrations of oleamide (0.1, 1, and 100 μM). On the basis of this experiment, we decided to perform only the inhibition assay of CYP2C6 (with diclofenac as a probe substrate). The kinetic parameters of CYP2C6 metabolic activity were determined using Michaelis-Menten kinetics. For construction of the Dixon plot and determination of the inhibition constant (Ki), diclofenac was used at concentrations of 4, 8, and 16 μM, and oleamide at 0, 10, 25, 37.5, and 50 μM.

2.8. CYP enzymatic activity assays

Human recombinant CYP3A4, CYP2B6, CYP2C9, and CYP1A2 enzymes expressed in a microsomal fraction of CYP cDNA baculovirus-infected insect cells (CYP450-Glo™ CYP3A4 Assay, CYP450-Glo™ CYP2B6 Assay, CYP450-Glo™ CYP2C9 Assay, and CYP450-Glo™ CYP1A2 Assay, Promega, Hercules, CA) were used to evaluate the interaction of oleamide with these enzymes in vitro according to the manufacturer's protocols. Ketoconazole, ticlopidine, fluconazole, and α-naphthoflavone were used as prototypical inhibitors of CYP3A4, CYP2B6, CYP2C9, and CYP1A2 enzymes.

2.8. Nuclear receptors and cell viability assays

2.8.1. Cell lines

A HepG2 human Caucasian hepatocyte carcinoma cell line was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, GBR) and was maintained in Dulbecco's

Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, USA).

2.8.2. Cell viability assay

To evaluate the toxicity of oleamide in HepG2 cells, a CellTiter 96® AQueousOne Solution Cell Proliferation Assay (Promega, Madison, USA) was performed according to the manufacturer´s protocol.

2.8.3. Plasmids

The construction of the PXR-responsive p3A4-luc construct containing XREM and basal promoter sequences and pSG5-hPXR expression construct have been described by Rulcova et al. (Rulcova et al., 2010). The AHR reporter plasmid (pXRE-luc) was described in our previous report (Dvorak et al., 2008). The pRL-TK construct was purchased from Promega (Madison, USA). The constructs pCAR-C/VP16 and pCAR-N/GAL4 for the CAR assembly assay have been described in a previous paper of ours (Carazo Fernandez et al., 2015).

2.8.4. Reporter gene assays (PXR, AhR)

All transient transfection gene reporter assays were performed with Lipofectamine® 3000 transfection reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA) in HepG2 cells. Briefly, cells were seeded onto 48-well plates and 24 h later were transfected with a luciferase reporter construct (150 ng/well), PXR expression plasmid (100 ng/well, for PXR experiments) and Renilla reniformis luciferase transfection control plasmid (pRL-TK) (30 ng/well). Then, the cells were stabilized for 24 h prior to treatment and further maintained in phenol red-free medium (150 µl) containing the evaluated compounds at the indicated concentrations for an additional 24 h. After treatment, the cells were lysed, and luciferase activity was measured (Dual Luciferase Reporter Assay, Promega, Madison, USA). Two modes were used as follows: (i) agonistic mode, in which the cells were treated with 0.1% DMSO or prototype ligand 5 µM or three concentrations of oleamide 1 or 10 or 30 µM. (ii) antagonistic mode, in which the cells were treated with 0.2% DMSO or with prototype ligands (5 µM) in combination with oleamide

at concentrations of 1 or 10 or 30 μM. Rifampicin (PXR activator) and 3-methylcholanthrene (AhR activator) were used as the prototype ligands. The data are expressed as a fold-change in firefly luciferase activity normalized to Renilla luciferase activity in each sample and relative to the vehicle (DMSO 0.1%)-treated control means, which were set to 1. Statistical analyses were done using at least three independent assays (n=3) performed in triplicate.

2.8.5. Assembly assay (CAR)

The CAR Ligand Binding Domain (LBD) assembly assay is based on two hybrid expression constructs encoding the C- (pCAR-C/VP16) and N- (pCAR-N/GAL4) terminal parts of human CAR LBD that are co-transfected together with the pGL5-luc luciferase reporter plasmid (Promega) containing UAS binding sites. A ligand promotes the connection of the two parts of the CAR LBD, resulting in luciferase transactivation. Transient transfection assays were carried out using Lipofectamine® 3000 transfection reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA) in HepG2 cells. Cells were seeded into 48-well plates and transfected with a pGL5-luc luciferase reporter construct (80 ng/well), the expression constructs pCAR-C/VP16 and pCAR-N/GAL4 (80 ng/well), and the Renilla reniformis luciferase transfection control plasmid (pRL-TK) (30 ng/well) 24 h later. Cells were maintained in a phenol red-free medium (150 μl) and treated with the tested compounds. After treatment, the cells were lysed, and luciferase activity was measured with a luciferase detection system (Dual Luciferase Reporter Assay, Promega). Two modes were used as follows: (i) agonistic mode, in which the cells were treated with 0.1% DMSO or CITCO (CAR activator) 5 μM or three concentrations of oleamide, 1 or 10 or 30 μM; and (ii) antagonistic mode, in which the cells were treated with 0.2% DMSO or CITCO 5 μM in combination with oleamide at concentrations of 1 or 10 or 30 μM. The data are expressed as the fold-change in firefly luciferase activity normalized to Renilla luciferase activity in each sample and relative to the vehicle (DMSO 0.1%) treated control means, which were set to 1. Statistical analyses were done using at least three independent assays (n=3) performed in triplicate.

2.8.6. Primary cultures of human hepatocytes and qRT-PCR

Human long-term hepatocytes in a monolayer (Batch HEP220971, Biopredic International, Saint Grégoire, France) were prepared from the livers of a 46-year-old Caucasian male. The medium was replaced with a serum-free medium the day after delivery of the primary human hepatocytes, and the cultures were allowed to stabilize for an additional 48 h prior to treatment. The cultures were maintained at 37 °C in 5% CO_2 in a humidified incubator and treated with oleamide (10 μM) together with prototype CAR (CITCO, 1 μM), PXR (rifampin, 10 μM) and AHR (3-methylcholantrene, 10 μM) ligands for 48 h before the total RNA isolation. cDNA synthesis and qRT-PCR analyses were performed as described previously (Rulcova et al., 2010).

2.9. Hormone assays

Serum concentrations of corticosterone were analysed by radio-immuno assay after dichloromethane extraction as described previously (Jezova et al., 1994). The intra- and inter-assay coefficient of variation values were 6% and 8% respectively, and the sensitivity of the assay was 0.5 μg/100 ml of plasma. The concentrations of free T3 and prolactin in serum were measured using commercially available ELISA kits (Rat Free T3 ELISA Kit and Prolactin rat ELISA, Cusabio Biotech Co., Ltd, USA, respectively). The intra- and inter-assay precision of the kits (coefficient of variation %) were less than 15%. The sensitivity and detection range reported by the manufacturer were 0.125 ng/ml and 0.125 ng/ml – 50 ng/ml for prolactin and 0.38 pmol/l and 2 pmol/l – 32 pmol/l for T3, respectively.

2.10. Data analysis

The results of the determination of CYP activities were statistically evaluated using either (i) parametric one-way ANOVA followed by Tukey's honestly significant difference test for unequal sample sizes with a normal distribution of data or (ii) non-parametric Kruskal-Wallis test followed by multiple comparisons of mean ranks for all groups with a non-normal distribution of data. The statistical evaluation of the results from western blot analyses was

performed using the Wilcoxon signed-rank test. One-way ANOVA with a Dunnett's post hoc test were used for data from nuclear receptor assays, human CYP inhibition assays, and primary hepatocytes assays. The non-parametric Mann–Whitney U-test was used to compare the hormone levels in two sets of samples. All values are expressed as means (SD) and they were computed using the software Statistica 13 (StatSoft, Inc. 2015). Results were considered statistically significant when $p \leq 0.05$. The Ki value was determined using a Dixon plot and calculated with the software SigmaPlot (SPSS, Inc., Chicago). The Michaelis-Menten parameters (Km and Vmax) and the kinetic pattern of inhibition were also assessed with SigmaPlot.

3. Results

3.1. Effect of oleamide on liver CYP activity in vivo

In preliminary experiments, we evaluated how propylene glycol (a vehicle of oleamide) influenced the total microsomal protein, CYP levels, and their metabolic activity. We found no statistical difference when compared to experiments with saline-treated animals (data not shown). Hence, propylene glycol-treated animals were further used as controls for oleamide-administered animals.

3.1.1. Total protein and total CYPs content

Animals administered with the highest dose of oleamide exhibited a significant decrease in the content in both total protein ($p < 0.05$, Kruskal-Wallis test, Fig. 1A) and CYP ($F_{(4,41)}$=4,89 $p < 0.01$, one-way ANOVA test, Fig. 1B) in RLMs compared to controls. The relative content of CYP, representing the ratio of cytochrome P450 to total protein content (nmol per mg of isolated protein in RLMs) was calculated, and again there was a significant decrease ($F_{(4,41)}$=4.89, $p < 0.01$, one-way ANOVA test) in the group treated with the highest dose of oleamide in comparison to the group treated with propylene glycol alone (Fig. 1C).

3.1.2. CYPs metabolic activity

The incubation of RLMs isolated from oleamide-treated animals with selective markers of CYP metabolic activity resulted in a variety of effects depending on the CYP isoform. None of the tested oleamide doses influenced the relative or specific metabolic activities of CYP2A and CYP2C6 (Fig. 2A, 2B and 3A, 3D respectively) (for definitions of “relative” and “specific” metabolic activity, see section 2.5). The rate of testosterone 2β and 6β-hydroxylation was decreased to 77% of the control value. Nevertheless, the change in CYP3A relative activity was not statistically significant (Fig. 2A). In contrast, the relative activities of the CYP1A ($F_{(4,41)}$=6,33 $p < 0.001$, one-way ANOVA test, Fig. 3B), CYP2B, CYP2C11 ($p < 0.01$, Kruskal-Wallis test, Fig. 2A), and CYP2D2 ($F_{(4,40)}$=7,42 $p < 0.001$, one-way ANOVA test, Fig. 3C) were significantly decreased in animals treated with the highest dose of oleamide. Lower doses

of oleamide were incapable of decreasing the relative metabolic activity of the CYPs. Specific metabolic activities with respect to the total content of CYP did not exhibit any differences between the oleamide-treated animals and controls in their CYP1A2 (Fig. 3E), CYP2D6 (Fig. 3F), or CYP2B (Fig. 2B). An exception was CYP2C11 (Fig. 2B), where the activities of both the 2α-, and 16α-hydroxylations of testosterone were decreased in the group treated with the highest dose of oleamide (one-way ANOVA test $F_{(4,39)}=8,22$ $p < 0.001$ and $F_{(4,41)}=5,31$ $p < 0.001$, respectively).

3.1.3. Content of distinct CYPs

The amount of distinct CYP proteins was determined only for CYPs with a significant change in the relative metabolic activity, with the exception of CYP2D2, because the monoclonal antibody was not available. In addition, the protein content was measured for CYP3A, where the decrease in metabolic activity was on the threshold of statistical significance. Blots were quantified, and the decrease in protein levels was present in all evaluated CYPs after the 10 mg/kg/day treatment with oleamide (Fig. 4). The most influenced enzyme was CYP1A2, with a drop in protein content to 33.6 ± 12% of the amount determined in the control group, followed by CYP3A (41.9 ± 12% of the control group). The amount of CYP2B and CYP2C11 decreased similarly to 54.5 ± 8% and 53.2 ± 1.3% of the control group, respectively.

3.2. The effect of oleamide in vitro

3.2.1. Rat CYP inhibition assays in vitro

The results of the pilot study when oleamide was incubated with RLMs from naïve animals showed that oleamide did not influence the activities of CYP2A, CYP2B, CYP3A, and CYP2C11 (Table 2). The metabolic activities of these enzymes ranged from 97 to 101% of the activity of control group in all analysed testosterone metabolites and at all concentrations of oleamide used. Nevertheless, oleamide weakly inhibited phenacetin *O*-deethylation indicating a weak inhibition of CYP1A2 activity. The rate of the reaction was suppressed to 77% of the rate of the control at a 100 μM concentration of oleamide in the incubation mixture. With

CYP2D2, it was similarly observed that the metabolic activity of the enzyme was suppressed up to 70% of the rate of the control at a 100 μM concentration of oleamide. Such a high concentration of any particular drug is usually clinically irrelevant. Together with the fact that the inhibition of metabolic activities was very weak, the Ki value was not assessed for CYP1A2 and CYP2D2.

CYP2C6 was the most sensitive CYP to the inhibitory effect of oleamide in the pilot study (Table 2). The kinetic parameters of CYP2C6 metabolic activity were determined by Michaelis-Menten kinetics (Km = 7.2 ± 0.94 μM, Vmax = 1.03 ± 0,04 nmol/min/mg protein). The 4′-hydroxylation of diclofenac was inhibited by 56% with a final concentration of oleamide in the incubation mixture of 100 μM. Thus, further studies to determine the type of inhibition were done. Competitive, non-competitive, and uncompetitive models of inhibition were evaluated and the respective Ki values were 28.7 μM, 71.6 μM, and 28.9 μM. The Akaike's Information Criterions corrected (AICc) in the individual models were used to evaluate the suitability of each model. The values of AICc were -652.591, -648.078, and -634.012 for the competitive, noncompetitive and uncompetitive models, respectively. Therefore, the competitive mode of inhibition seems to be an appropriate model with the best fit (Fig. 5).

3.2.2. Nuclear receptors and cell viability assays

Oleamide was tested at a range of concentrations from 1-30 μM for interaction with PXR, CAR, and AhR in HepG2 cells by gene reporter assays. No significant activation by the compound was observed for any of these receptors (Fig. 6). Consistently, we did not observe inhibition of these nuclear receptors in antagonistic experiments with prototype PXR, CAR, and AhR ligands (rifampicin, CITCO, 3-methoxychlorantrene, respectively) (Fig. 6).

In addition, potential cytotoxicity was studied with the CellTiter 96® assay in HepG2 cells. The results clearly showed that oleamide is not toxic at the highest concentration assayed (30 μM) in HepG2 cells (data not shown).

3.2.3. Human CYP inhibition assays and induction experiments in primary human hepatocytes by oleamide

In the next set of experiments, we studied whether oleamide could inhibit human CYP2B6, CYP2C9, CYP3A4, and CYP1A2 enzymes in vitro and if it could induce key CYP genes regulated by the xenobiotic nuclear receptors CAR, PXR, and AHR.

We found that oleamide inhibits none of tested human CYPs up to a concentration of 30 μM (Fig. 7A-D). With CYP2B6, we observed an insignificant inhibition of metabolic activity at a 30 μM concentration of oleamide (Fig. 7A.).

We then treated primary human hepatocytes with 10 μM oleamide and CAR (CITCO, 1 μM), PXR (rifampicin, 10 μM), and AhR (3-methylcholantrene, 10 μM) ligands. We observed a significant induction of CYP2B6 mRNA by CITCO, CYP3A4 mRNA by rifampicin, and CYP1A2 mRNA by 3-methylcholantrene after 48 h of treatment (Fig. 7E). Oleamide slightly upregulated all CYP2B6, CYP3A4, and CYP1A2 mRNA (2.0, 2.5 and 3.3-fold, respectively), but the effects were not statistically significant.

3.3. Serum hormone concentrations

There were no statistical differences between control and oleamide treated animals in serum levels of any of the hormones measured (Fig. 8).

4. Discussion

In this study, we demonstrate the effect of the endocannabinoid oleamide on the rat liver content and activities of key liver CYPs, namely CYP1A2, CYP2A, CYP2B, CYP2C, CYP2D2, and CYP3A. The in vivo effect was assessed after the repeated administration of three different doses of oleamide to rats (0.1, 1, and 10 mg/kg/day). The doses were selected with respect to available data for oleamide biological activity including hypnogenic (10-20 mg/kg, i.p.) (Huitron-Resendiz et al., 2001), analgesic (ED_{50}=66 mg/kg, i.p.) (Fedorova et al., 2001), hypothermic (ED_{50}=14 mg/kg, i.p.; 2.5-20 mg/kg, i.p, respectively) (Fedorova et al., 2001; Huitron-Resendiz et al., 2001), anxiolytic (5 mg/kg, i.p.) (Fedorova et al., 2001), and antidepressant-like (5 mg/kg, i.p.) (Ge et al., 2015) effects in animal experiments. Our results showed that oleamide at a dose of 10 mg/kg/day decreased the total protein content as well as total CYP protein levels in liver (Fig. 1). However, the metabolic activities of the individual tested CYPs varied (Fig. 2, 3). The overall decrease in the relative metabolic activity could be interpreted in two ways. It could be caused by an inhibition of CYP enzymes in terms of a decreased ability to catalyse the conversion of substrate to the appropriate metabolite with the preserved amounts of enzyme. The other possibility is the downregulation of CYPs – meaning decreased amounts of distinct CYP proteins with the preserved metabolic activity. In our study, the relative metabolic activities of CYPs were assessed after the standardization per total protein content, but not per individual amount of CYPs. Decreased levels of distinct CYPs (Fig. 4) corresponded to their decreased relative metabolic activities (Fig. 2, 3). When relative metabolic activities were recalculated to specific metabolic activities with respect to the total CYP content in the incubation mixtures, there were no significant changes in the rate of marker reactions between the tested groups, except for the decreased activity of CYP2C11 with the highest dose of oleamide (Fig. 2). The reduction in CYP2C11 activity was higher than could be explained by a simple decrease in total CYP content, but the exact mechanism of this influence is still under investigation. Based on the experimental data, we concluded that the metabolic activity of most enzymes in the in vivo experiment had not been affected. The decreased relative

activities measured in our experiment were caused by CYP downregulation rather than by inhibition of enzymes, which is in line with the results of western blot testing.

The effects of other endocannabinoids on the activity of CYPs are unknown, and the influence of exogenous cannabinoid receptor ligands on CYPs is diverse. In detail, the decrease in the microsomal protein content in experimental animals was reported after administration of the CB_1 ligand (CP 55,940) to mice (Fontanellas et al., 2005) and rats (List et al., 1977; Dalterio et al., 1986; Costa et al., 1996). Cannabidiol also decreased the microsomal protein levels in human HepG2 cells (Yang et al., 2014) and inhibited metabolism of the selected CYP substrates in mice (Watanabe et al., 1981; Bornheim and Correia, 1989). On the other hand, studies suggesting that cannabinoids (CP55,940, hashish, and cannabidiol) would increase the content and/or activity of liver CYPs in rodents have been published (Dalterio et al., 1986; Costa et al., 1996; Sheweita, 2003). The fact that these substances are usually of a different chemical structure with different biological activity limits the generalization of the results obtained using different cannabinoids as a general "class effect" of cannabinoids. Moreover, the ECS could have reacted differently to the same drug at different time points during drug administration, as mechanisms of desensitization, or receptor down- or upregulation might have been involved (Daigle et al., 2008; Burston et al., 2010).

The ECS, as a retrograde modulator, is associated with different neuronal systems within the brain, and therefore it can change the activities of several neurotransmitters in various brain regions. It was documented that changes in the activity of these specific neuronal circuits could influence the activity of liver CYPs. Hormones were identified as a probable link between the brain and liver (Wojcikowski et al., 2008; Wojcikowski and Daniel, 2011; Bromek et al., 2013; Rysz et al., 2016). While exogenous cannabinoids have been shown to be modulators of pituitary hormones for a long time (Pagotto et al., 2006), the influence of endocannabinoids on the neuroendocrine system has not been fully understood. Endocannabinoids modulate the levels of growth hormone (Pagotto et al., 2006), thyrotropin-releasing hormone (Deli et al., 2009), corticotrophin-releasing hormone (Steiner and Wotjak, 2008; Hill and Tasker, 2012),

prolactin, and luteinizing hormone (de Miguel et al., 1998), probably via the hypothalamus, where the ECS acts as a retrograde messenger system. Hormones then bind to nuclear receptors in hepatocytes and regulate CYP gene expression. Therefore, the ECS is probably involved in the regulation of the metabolic activity of liver CYPs as well. This hypothesis was described in details elsewhere (Zendulka et al., 2016). In our study, oleamide did not influence the levels of prolactin, corticosterone, or free triiodothyronine (Fig. 8). Therefore, we expect these hormones to not be included in the mechanism of liver CYP activity being influenced by oleamide.

The interactions of oleamide with the key human nuclear receptors involved in regulation of major CYPs were further tested to obtain additional data on the possible influence of this endocannabinoid in humans. The results showed that oleamide did not interact with human AhR, PXR, or CAR nuclear receptors either in an agonistic or antagonistic mode (Fig. 6). Consistently, oleamide did not induce CYP2B6, CYP2C9, or CYP3A4 mRNA expression in a representative primary human hepatocyte culture (Fig. 7E).

The direct inactivation of rat CYPs by oleamide in RLMs was tested in our in vitro model. Weak inhibition of CYP1A2, CYP2D2, and more noticeably of CYP2C6 were described (Table 2). Similarly, the direct inhibition of CYPs by phytocannabinoids, such as cannabidiol, cannabinol, and Δ^9-THC were reported (Zendulka et al., 2016). All three substances inhibited the metabolic activity of CYPs with various potencies. Differences between the results obtained from two models used in our study can be explained by the divergent modes of how the metabolic activity of CYPs can be influenced. In an in vitro experiment, the drug could have bound either to the active or allosteric site of CYPs and decreased the metabolic activity by direct enzyme inactivation. Livers isolated from animals treated with oleamide were drawn 24 h after the last dose of drug was administered and microsomes were washed out several times with buffers during the process of isolation. Therefore, the microsomes were thought to be "drug free". This was proved by there being no change in the metabolic activity of CYP2C6 detected in the in vivo model compared to the results of the in vitro model, where CYP2C6 was inhibited, and conversely by a decreased metabolic activity of CYP2C11 assessed in vivo, with

no influence in vitro. Furthermore, the direct interaction with enzymes could not explain the decreased CYP protein levels, which were detected for CYP1A2, CYP2B, CYP2C11, and CYP3A after a repeated systemic administration of oleamide (Fig. 2-4). These findings further support our conclusion that the influence of oleamide on rat CYPs was mediated by the mechanism of downregulation. Besides the direct interaction of oleamide with RLMs we also tested its interaction with human recombinant CYPs (CYP2B6, CYP2C9, CYP3A4, and CYP1A2), finding significant effects (Fig. 7A-D).

In conclusion, we hypothesize that the repeated i.p. administration of oleamide (10 mg/kg/day) caused the downregulation of CYP1A2, CYP2B, CYP2C11, and CYP2D2 in rats. The effect is unlikely to be caused by the direct inhibition of CYP enzymes, or by oleamide influencing the hormonal levels. The mechanism of this effect has not been determined yet. While, oleamide inhibited rat CYP1A2, CYP2D2, and CYP2C6 enzymes, it did not interact with the tested recombinant human CYPs in vitro. No effect on human AhR, PXR, and CAR nuclear receptors was observed in the regulation of main human CYPs in the primary human hepatocytes. To the best of our knowledge, we are the first to report the effects of the endocannabinoid CB_1 agonist and the FAAH inhibitor, oleamide, on rat and human CYPs. Our results suggest the possible involvement of the endocannabinoid system in the regulation of rat liver CYPs activity. Still, oleamide affected neither the activity of human recombinant CYPs nor the expression of mRNA of CYPs in human primary hepatocytes. The effects observed in rat CYP enzymes are likely to be species-specific, and its clinical impact needs to be elucidated in future studies.

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Conflict of Interest

The authors declare no conflicts of interest.

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Footnote

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Legends for Figures

Fig. 1. Changes in total protein content (**A**), total CYP content (**B**) and relative content of cytochrome P450 (**C**) (nmol/mg of microsomal protein) after systemic administration of oleamide. The total protein content in individual rat liver microsomes was assessed according to the method of Lowry et al., and the total CYP content was determined using a CO difference spectroscopy method according to Omura et al. Animals were treated i.p. with oleamide 0.1 (n=10), 1 (n=10), 10 mg/kg/day (n=10), and propylene glycol (control group, n=6). Bars and whiskers express the mean (SD). Statistical significance was assessed by Kruskal-Wallis test followed by multiple comparisons of mean ranks (A, C) and one-way ANOVA test followed by Tukey's honestly significant difference test for unequal sample sizes (B). Statistical significance with respect to the control group (propylene glycol) is indicated with $*p \leq 0.05$.

Fig. 2. A) Relative metabolic activities of CYP2A, CYP2B, CYP3A, and CYP2C11 after systemic administration of oleamide, measured as rate of testosterone hydroxylation and shown as % of rate of reaction in control group. The absolute control values (pmol/min/mg of total protein content) were as follows: 217.4 ± 23.7, 37.5 ± 6.1, 76.2 ± 14.8, 614.8 ± 92.0, 3303.0 ± 779.0, and 2934.5 ± 791.6 (testosterone 7α-, 16β-, 2β-, 6β-, 2α-, and 16α-hydroxylations, respectively). Animals were treated i.p. with oleamide 0.1 (n=10), 1 (n=10), 10 mg/kg/day (n=10), and propylene glycol (control group, n=6). Reactions were performed in the presence of testosterone (400 μM) in RLMs (1 mg/ml of the total protein content) with an NADPH generating system, in a final volume of 1 ml at 37 °C for 20 min. **B)** Specific metabolic activities of CYP2A, CYP2B, CYP3A, and CYP2C11 after systemic administration of oleamide measured as rate of testosterone hydroxylation per nmol of total CYP content per minute, and shown as % of rate of reaction in control group. The absolute control values (pmol/min/nmol of total CYP content) were as follows: 350.8 ± 38.8, 60.3 ± 8.2, 122.5 ± 20.2, 989.5 ± 127.0, 5321.8 ± 1188.7, and 4726.4 ± 1209.4 (testosterone 7α-, 16β-, 2β-, 6β-, 2α-, and 16α- hydroxylations, respectively). All values are expressed as bar graphs of the mean (SD).

Statistical significance was assessed by the Kruskal-Wallis test followed by multiple comparisons of mean ranks (A) and one-way ANOVA test (B). Statistical significance with respect to the control group (propylene glycol, n=6), is indicated with **$p \leq 0.01$.

Fig. 3. A) Relative metabolic activity of CYP2C6 after systemic administration of oleamide, measured as rate of diclofenac 4′-hydroxylation and shown as % of rate of a reaction in the control group. The absolute control value was 1194.4 ± 184.4 pmol/min/mg of total protein content. **B)** Relative metabolic activity of CYP1A2 after systemic administration of oleamide, measured as rate of phenacetin *O*-deethylation and shown as % of rate of a reaction in the control group. The absolute control value was 361.3 ± 21.5 pmol//min/mg of total protein content. **C)** Relative metabolic activity of CYP2D2 after systemic administration of oleamide, measured as rate of dextromethorphan *O*-demethylation and shown as % of rate of a reaction in the control group. The absolute control value was 1154.9 ± 135.0 pmol//min/mg of total protein content. Animals were treated i.p. with oleamide 0.1 (n=10), 1 (n=10), 10 mg/kg/day (n=10), and propylene glycol (control group, n=6). Reactions were performed in the presence of diclofenac (100 μM), phenacetine (400 μM) or dextromethorphan (500 μM) in RLMs (1 mg/ml of the total protein content) with the NADPH generating system, in a final volume of 1 ml at 37 °C for 15 min (phenacetine) or 20 min (diclofenac, and dextromethorphan). **D, E, F)** Specific metabolic activities of CYP2C6, CYP1A2, and CYP2D6 after systemic administration of oleamide measured as rate of testosterone hydroxylation per nmol of total CYP content per minute and shown as % of the rate of reaction in control group. The absolute control values (pmol/min/nmol of total CYP content) were as follows: 1926.3 ± 292.4, 582.48 ± 23.4, and 1859.5 ± 169.2 (diclofenac 4′-hydroxylation, phenacetin *O*-deethylation, and dextromethorphan *O*-demethylation, respectively). Bars and whiskers express the mean (SD). Statistical significance was assessed by one-way ANOVA test followed by Tukey's honestly significant difference test for unequal sample sizes. Statistical significance with respect to the control group (propylene glycol, n=6), is indicated with ***$p \leq 0.001$.

Fig. 4. Western blot analysis showing the expression of CYP1A2, CYP2B, CYP2C11, and CYP3A1 enzymes in rat liver microsomes after systemic administration of oleamide. Animals were treated i.p. with oleamide 0.1 (n=10), 1 (n=10), 10 mg/kg/day (n=10), and propylene glycol (control group, n=6). **A)** Representative blots of three individual animals of each group show expression of each indicated protein (10 μg in all cases). **B, C, D, E)** Bar graphs show quantification of blots for CYP1A2, CYP2B, CYP2C11, and CYP3A1 protein, respectively. The bars represent means (SD) of individual animals of each group in three repetitions. Blots were quantified relative to the loading control (β-actin). Statistical significance was assessed by Wilcoxon signed-rank test and is shown as $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ when compared to the control.

Fig 5. Dixon plot of the effect of oleamide on 4′-hydroxydiclofenac formation in rat liver microsomes (RLMs) from naïve animals in a competitive model of inhibition. Reactions were performed in the presence of diclofenac (4, 8, 16 μM) and various concentrations of oleamide (0, 10, 25, 37.5, 50 μM) in RLMs (0.25 mg/ml of the total protein content) with the NADPH generating system in a final volume of 1 ml at 37 °C for 30 min after 10 min pre-incubation. The reaction was performed in triplicates. V = velocity of the reaction (nmol of 4ˈhydroxydiclofenac /min/ mg protein) and I = concentration of oleamide (μM).

Fig 6. Interaction of oleamide with pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR), or constitutive androstane receptor (CAR) in luciferase assays in HepG2 cells in agonistic (**A, C, E**) and antagonistic (**B, D, F**) modes. In the agonistic mode, cells were treated with 0.1% DMSO (control) or with prototype ligands (5 μM) or three concentrations of oleamide 1 or 10 or 30 μM. In the antagonistic mode, cells were treated with prototype ligands (5 μM) in combination with oleamide at concentrations of 1 or 10 or 30 μM. Rifampicin (RIF, PXR activator), 3-methylcholanthrene (3-MC, AHR activator), and CITCO (CAR activator)

were used as prototype ligands. The data are expressed as the fold change in firefly luciferase activity normalized to Renilla luciferase activity in each sample and relative to the vehicle (DMSO)-treated control means, which were set to 1. Statistical analyses were performed using at least three independent assays (n=3) performed in triplicate and statistical significance was assessed by one-way ANOVA followed by Dunnett's test.

Fig. 7. Oleamide does not affect CYP2B6, CYP2C9, CYP3A4, and CYP1A2 enzymatic activities in vitro and mRNA expression in primary human hepatocytes. Oleamide does not inhibit metabolic activities of human CYP2B6 (**A**), CYP2C9 (**B**), CYP3A4 (**C**), and CYP1A2 (**D**) enzymes expressed in the microsomal fraction of CYP cDNA baculovirus-infected cells. Assays were performed in three independent experiments (n=3) measured in triplicate. (**E**) qRT-PCR experiment in primary human hepatocytes treated with oleamide (10 μM), CITCO, rifampicin (RIF) or 3-methylcholantrene (3-MC) for 48 hours before total RNA isolation and qRT-PCR analysis were done. Data are presented as the means (SD) of triplicates from a representative batch of primary human hepatocytes (human long-term hepatocytes in monolayer, Batch HEP220971, Biopredic) and are expressed as the fold mRNA upregulation relative to the vehicle-treated controls (set to 100%). Statistical significance was assessed by one-way ANOVA, *$p \leq 0.05$, ***$p \leq 0.001$.

Fig 8. Effect of systemic administration of oleamide on hormone concentrations in rat blood serum. Animals were treated i.p. with oleamide 10 mg/kg/day (n=10), and propylene glycol (control group, n=6). Bar graphs show concentrations of free triiodothyronine (**A**), prolactin (**B**), and corticosterone (**C**). The bars represent means (SD) of individual animals of each group in three repetitions. Statistical significance was assessed by Mann-Whitney U-test.

Table 1. Primary and secondary antibodies used for Western blotting

Name of antibody	**Biological source**	**Species**[a]	**Catalogue Number**	**Company**	**Dilution**
Anti-CYP1A2	mouse	m, r, h	sc-53241	Santa Cruz[b]	1:500
Anti-CYP2B1/2B2	mouse	m, r	sc-73546	Santa Cruz	1:500
Anti-CYP2C11	goat	r	R-PAP 121	Cypex[c]	1:5,000
Anti-CYP3A1	mouse	m, r, h	sc-53246	Santa Cruz	1:1,500
Anti-β-actin	rabbit	m, r, h, b,	sc-4970	Cell Signaling[d]	1:500
Anti-mouse	goat		A4416	Sigma-Aldrich[e]	1:5,000
Anti-goat	rabbit		A5420	Sigma-Aldrich	1:5,000
Anti-rabbit	goat		A6667	Sigma-Aldrich	1:5,000

[a] m=mouse, r=rat, h=human, b=bovine, p=pig, k=monkey; [b] Dallas, USA; [c] Dundee, GBR; [d] Danvers, USA; [e] St. Louis, USA

Table 2. CYPs interactions with oleamide in vitro (data from the pilot experiment)

	CYP metabolic activity (% activity of control)								
Oleamide	1A2	2C6	2D2	2A	2B	2C11		3A	
0.1 μM	99.7	110.5	110.0	99.9[a]	100.0[b]	100.1[c]	99.6[d]	100.0[e]	100.1[f]
1 μM	108.6	94.2	96.8	100.1[a]	100.0[b]	100.8[c]	100.6[d]	100.0[e]	100.2[f]
100 μM	77.4	44.2	70.4	98.4[a]	99.9[b]	100.9[c]	99.4[d]	100.0[e]	100.0[f]

The metabolic activities of CYP2A, CYP2B, CYP3A, and CYP2C11 were measured as the rate of testosterone hydroxylation. The absolute control values (pmol/mg protein/min) were as follows: 1866.7 ± 0.9, 1773.7 ± 0.4, 2080.4 ± 3.5, 2123.9 ± 5.0, 1773.6 ± 0.8, 1825.6 ± 1.3 (testosterone [a] 7α-, [b] 16β-, [c] 2α-, [d] 16α-, [e] 2β-, and [f] 6β- hydroxylations, respectively). The metabolic activity of CYP1A2 was measured as the rate of phenacetin *O*-deethylation and control value 53.3 ± 1.2 pmol/mg protein/min. The metabolic activity of CYP2C6 was measured as the rate of diclofenac 4′-hydroxylation and control value 203.8 ± 11.3 pmol/mg protein/min. The metabolic activity of CYP2D2 was measured as the rate of dextromethorphan *O*-demethylation and control value 320.2 ± 7.6 pmol/mg protein/min. Experiments were performed with single concentrations of substrates phenacetin (300 μM), testosterone (100 μM), diclofenac (8 μM), or dextromethorphan (28 μM), and three concentrations of oleamide in rat livers microsomes from naïve animals (0.25 mg/ml of the total protein content) with the NADPH generating system in a final volume of 1 ml at 37 °C. After 10 min of pre-incubation with a different concentration of oleamide, reactions were initiated by addition of specific markers. The incubation times were 40 min (phenacetin and dextromethorphan), 20 min (testosterone), and 30 min (diclofenac). All reactions were performed in triplicates.

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Fig. 1

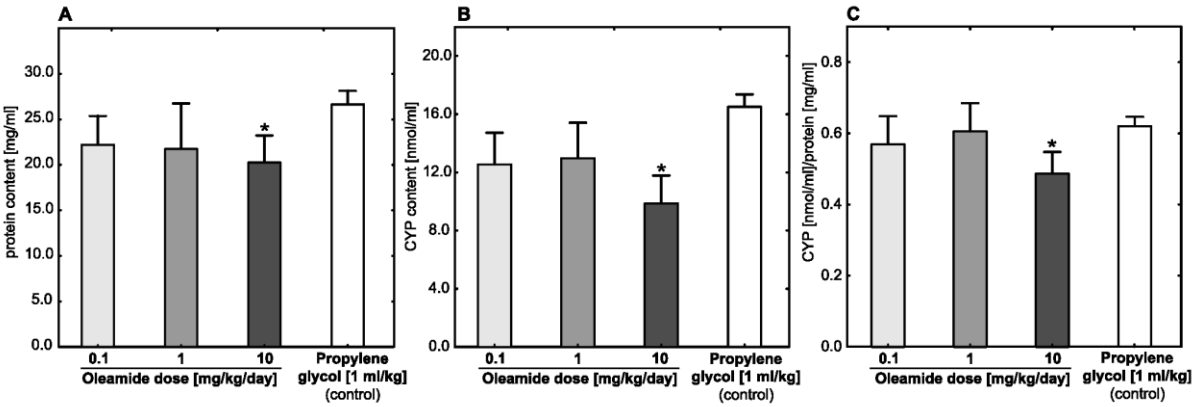

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Fig. 2

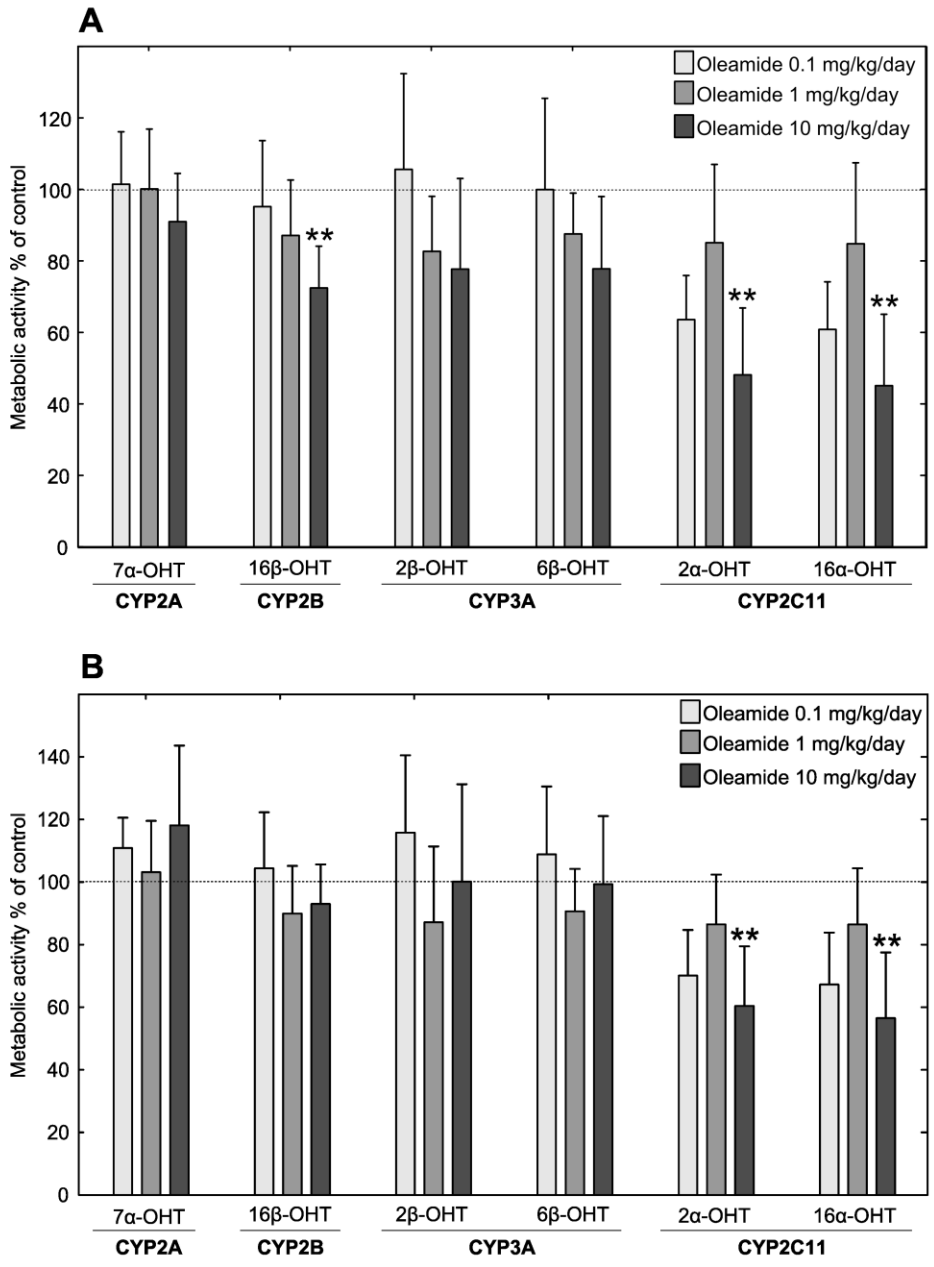

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Fig. 3

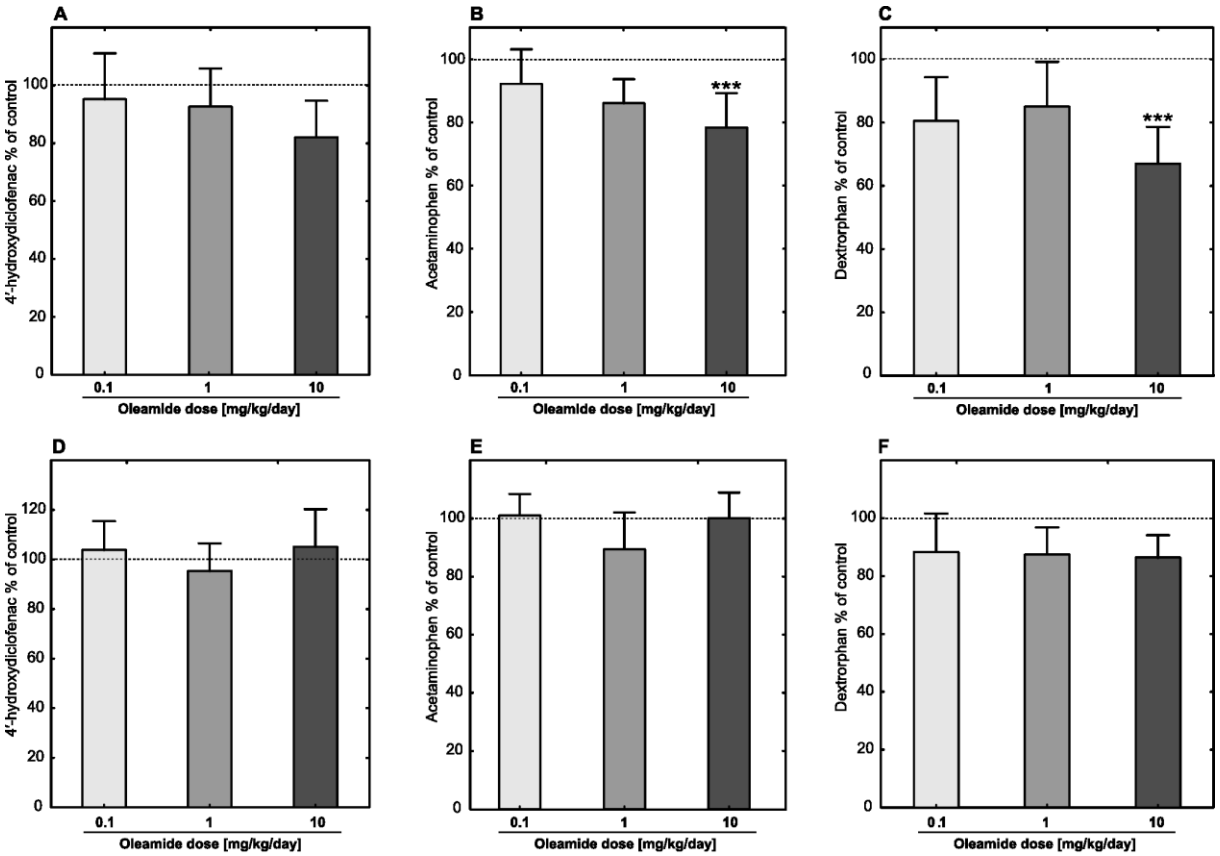

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Fig. 4

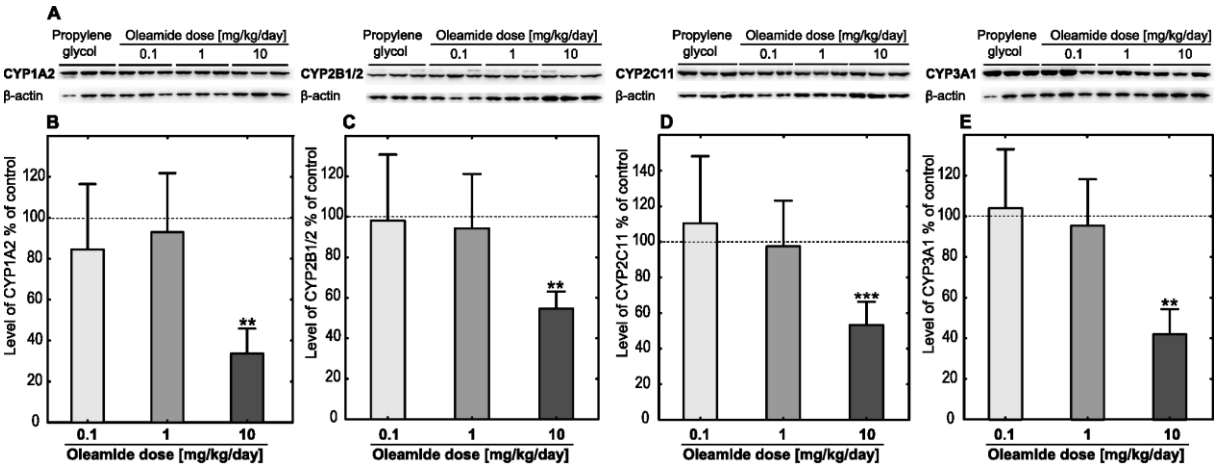

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Fig. 5

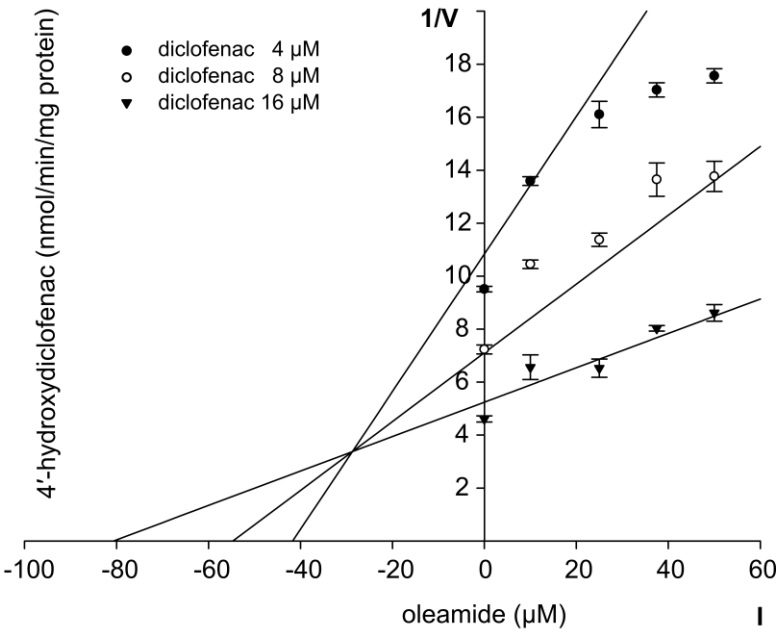

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Fig. 6

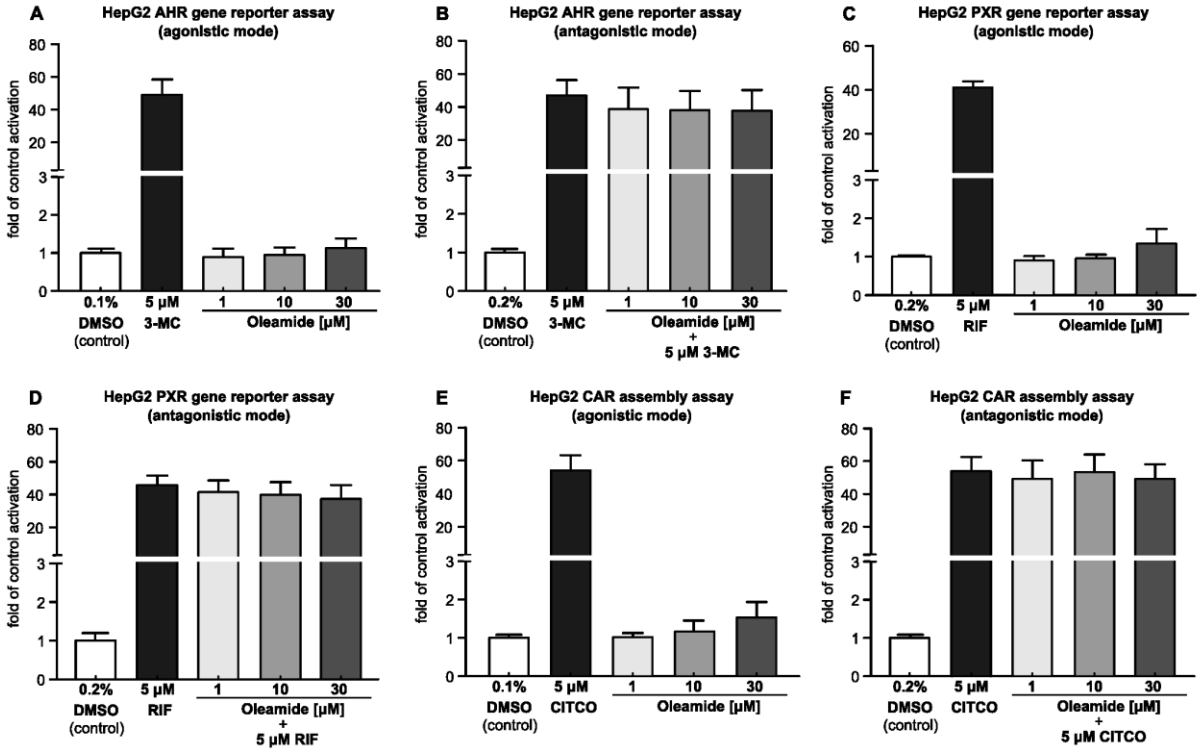

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Fig. 7

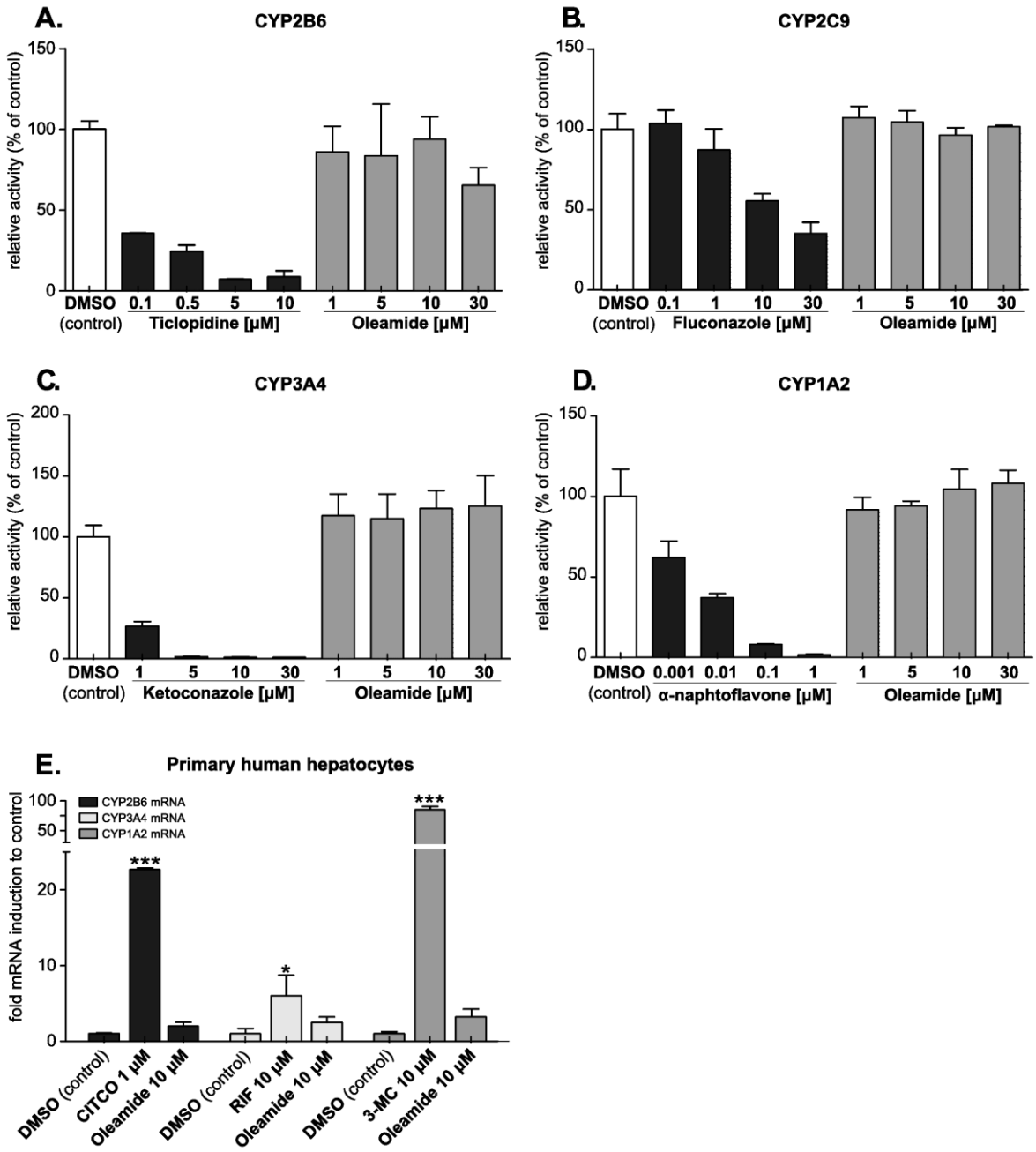

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Fig. 8