Robust Hydrolysis of Prostaglandin Glycerol Esters by Human Monoacylglycerol Lipase (MAGL)

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ABSTRACT

The primary route of inactivation of the endocannabinoid 2-arachidonoylglycerol in the central nervous system is through enzymatic hydrolysis, mainly carried out by monoacylglycerol lipase (MAGL), along with a small contribution by the α/β-hydrolase domain (ABHD) proteins ABHD6 and ABHD12. Recent methodological progress allowing kinetic monitoring of glycerol liberation has facilitated substrate profiling of the human endocannabinoid hydrolyses, and these studies have revealed that the three enzymes have distinct monoacylglycerol substrate and isomer preferences. Here, we have extended this substrate profiling to cover four prostaglandin glycerol esters, namely, 15-deoxy-12,14-prostaglandin J2-2-glycerol (15d-PGJ2-G), PGD2-G, PGE2-G, and PGF2a-G. We found that the three enzymes hydrolyzed the tested substrates, albeit with distinct rates and preferences. Although human ABHD12 (hABHD12) showed only marginal activity toward PGE2-G, hABHD6 (hABHD6) preferentially hydrolyzed PGD2-G, and human MAGL (hMAGL) robustly hydrolyzed all four. This was particularly intriguing for MAGL activity toward 15d-PGJ2-G whose hydrolysis rate rivaled that of the best monoacylglycerol substrates. Molecular modeling studies combined with kinetic analysis supported favorable interaction with the hMAGL active site. Long and short MAGL isoforms shared a similar substrate profile, and hMAGL hydrolyzed 15d-PGJ2-G also in living cells. The ability of 15d-PGJ2-G to activate the canonical nuclear factor erythroid 2-related factor (Nrf2) signaling pathway used by 15d-PGJ2 was assessed, and these studies revealed for the first time that 15d-PGJ2 and 15d-PGJ2-G similarly activated Nrf2 signaling as well as transcription of target genes of this pathway. Our study challenges previous claims regarding the ability of MAGL to catalyze PG-G hydrolysis and extend the MAGL substrate profile beyond the classic monoacylglycerols.

Introduction

The endocannabinoids anandamide (N-arachidonoylthetanalamide, AEA) and 2-arachidonoylglycerol (2-AG) are ω-6 arachidonic acid (AA)-containing lipids involved in a broad range of processes, including synaptic transmission, food intake, energy balance, pain, and inflammation (Di Marzo et al., 2007; Kano et al., 2009; Alhouyek et al., 2014). The endocannabinoids signal via two G protein–coupled receptors (cannabinoid type 1 receptor [CB1R] and type 2 receptor [CB2R]) that show unique and tissue-specific distribution. Cannabinoid CB1R is widely expressed in the nervous system, mainly in axonal terminals of central and peripheral neurons whereas CB2R is principally expressed in cells of the immune system. Besides engaging G protein–coupled receptors, recent evidence implicates members of the transient receptor potential (TRP) ion channel family as ionotropic cannabinoid receptors (Caterina 2014). The signaling function of anandamide is primarily terminated by fatty acid amide hydrolase (FAAH) with AA and ethanolamine formed as the products. The major route for 2-AG inactivation is via enzymatic hydrolysis, liberating AA and glycerol. At the bulk brain level in vitro, MAGL accounts for ~85% of this hydrolysis, and the remaining ~15% is chiefly attributed to ABHD6 and ABHD12, two recently discovered...
alpha/beta-hydrolase domain (ABHD) containing serine hydrolases (Blankman et al., 2007; Savinainen et al., 2012).

Recent studies have indicated that in certain tissues such as the brain MAGL acts as a key metabolic node capable of linking together the endocannabinoid and the prostaglandin (PG) lipid signaling pathways (Mulvihill and Nomura 2013). This is because MAGL-catalyzed 2-AG hydrolysis generates AA, the key substrate for cyclooxygenase-2 (COX-2)–dependent oxygenation to produce eicosanoids such as prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2). In animal models of neurodegenerative diseases, MAGL-driven 2-AG hydrolysis has been shown to feed the principal substrate for COX-2–derived neuroinflammatory prostaglandins. Moreover, both genetic and pharmacologic MAGL inhibition has been found to exert anti-inflammatory and neuroprotective effects (Nomura et al., 2011; Chen et al., 2012; Prio et al., 2012). Thus, MAGL inhibitors may have therapeutic potential.

Comprehensive MAGL inactivation drastically alters brain lipid composition, most notably elevating levels of 2-AG and concomitantly decreasing those of AA. The resulting 2-AG overflow brings about functional antagonism of the endocannabinoid system (Chanda et al., 2010; Schlosburg et al., 2010). Under such conditions, 2-AG spillover might be increasingly canalized toward alternative metabolic pathways such as oxidation, catalyzed by COX, lipoxigenases (LOX), or cytochrome P450 family members (Rouzer and Marnett 2011). In particular, COX-2–mediated oxygenation is emerging as the third major metabolic pathway for endocannabinoids with potential therapeutic implications (Hermanson et al., 2014). COX-2–catalyzed 2-AG oxidation is thought to generate prostaglandin glycerol esters (PG-G) via pathways closely matching those of the respective prostaglandins formed from AA (Rouzer and Marnett 2011; Hermanson et al., 2014). Indeed, recent studies have demonstrated that under specific conditions, 2-AG–derived PG-G species are formed by COX-2 action in vitro and in vivo and that these metabolites likely have bioactivities of their own, mediated via as-yet-unidentified non-PG receptors (Rouzer and Marnett 2011; Hermanson et al., 2014).

In this scenario, the lifetime and signaling function of the PG-G species would be expected to be under tight enzymatic control, yet very little is known on the metabolic fates of PG-G in vivo or in vitro. The prevailing view is that rapid hydrolysis of PG-G is performed by unknown enzymatic activity, distinct from the endocannabinoid hydrolases fatty acid amide hydrolase (FAAH) and MAGL (Rouzer and Marnett, 2011). This view relies on the findings of a single study reporting that PGE2-G and PGF2a-G were poor substrates for the purified rat MAGL (Vila et al., 2007). However, our recent studies and those conducted in other laboratories are questioning the validity of this view by demonstrating that human MAGL efficiently hydrolyzed various PG-G species (Xie et al., 2010; Laitinen et al., 2014). While extending the substrate repertoire to cover glycerolipid substrates beyond the classic monoacylglycerol (MAG) species, we observed that human endocannabinoid hydrolases readily used PG-G species, albeit with distinct preferences and catalytic rates. This was particularly evident for human MAGL (hMAGL), for which 15d-PGJ2-G was found to be among the best in vitro substrates identified to date. The initial observations were recently reported as part of a study characterizing the role of conserved cysteine residues in the MAGL substrate preference and inhibitor profile (Laitinen et al., 2014). Here, we report our findings regarding hMAGL-catalyzed hydrolysis of PG-G species in more detail.

**Materials and Methods**

**Drugs, Chemicals, and Reagents.** All components of the glycerol assay mix (Navia-Paldanius et al., 2012), as well as 1-DG (1-decanoyl-rac-glycerol) were purchased from Sigma-Aldrich (St. Louis, MO). We synthesized 1(3)-AG in our laboratory or bought it from Cayman Chemicals (Ann Arbor, MI). We purchased 2-AG, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), and 15-deoxy-Δ12,14-prostaglandin J2-glycerol (15d-PGJ2-G) from Cayman Chemicals. The chemical structures and calculated logP values of the PG-G species are shown in Supplemental Fig. 1. While 15d-PGJ2-G represented the 2-isomer, PGD2-G, PGF2-G, and PGF2a-G were 1(3)-isomers. The MAGL inhibitor JKKK-048 [4-[bis-(benzo[d][1,3]dioxol-5-yl)methyl]-piperidin-1-yl] (1H-1,2,4-triazolo-1-yl)methanone was synthesized as previously described elsewhere (Aaltomaa et al., 2013).

**Human Embryonic Kidney 293 Cells with Transient Over-expression of Serine Hydrolases.** Our procedures have been described elsewhere (Navia-Paldanius et al., 2012); briefly, human embryonic kidney 293 (HEK293) cells were cultured as monolayers in Dulbecco’s modified Eagle’s medium (DMEM) (Euroclone, Milan, Italy) containing 10% fetal bovine serum (Euroclone) under antibiotics (penicillin/streptomycin, Euroclone) at 37°C in a humidified atmosphere of 5% CO2/95% air. All assays were performed in triplicate. All plasmids were purchased from Origene Technologies (Rockville, MD). Plasmid for the short isoform of hMAGL was generated by point-mutating the start codon (Met1Leu) of the long isoform by using the Stratagene QuickChange Site-Directed Mutagenesis Kit (La Jolla, CA). Plasmids were introduced to cells by a standard transient transfection procedure using the X-tremeGENE Hp DNA Transfection reagent (Roche, Mannheim, Germany). HEK293 and/or Mock cells (cells transfected with an empty vector) were cultured in parallel for controlling expression and activity in later experiments. Cellular lysates were prepared and protein concentrations measured as previously described elsewhere (Navia-Paldanius et al., 2012). In studies with intact cells, a stable hMAGL-transfected HEK293 cell line was used. The generation and culturing of this line has been previously described elsewhere (Savinainen et al., 2010).

**Human Embryonic Umbilical Vein Endothelial Cells.** Human embryonic umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords (unspecified sex) obtained from the maternity ward of the Kuopio University Hospital, after approval by the Kuopio University Hospital ethics committee. Cells were cultivated as previously described elsewhere (Levonen et al., 2001).

**Hydrolase Assays.** Hydrolase activity assays for hABHD6, hABHD12, and hMAGL, based on fluorescent glycerol detection were conducted in a 96-well-plate format as previously described elsewhere (Navia-Paldanius et al., 2012). The amount of lysate protein per well was 0.3 or 1 µg for HEK293 cells and HUVEC, respectively. The substrate preferences were determined in TEMN-BSA buffer (50 mM Tris-HCl pH 7.40, 1 mM EDTA, 5 mM MgCl2, 100 mM NaCl, supplemented with 0.5% (w/v) fatty acid free bovine serum albumin and the test substrates (5 or 25 µM final concentration, or 3–50 µM in assays for Km determination). For each tested substrate, assay blanks without enzyme, cellular background (HEK293/Mock cell lysates), and a glycerol standard was included to monitor assay performance. Fluorescence of the assay blank was subtracted from all values before calculation of the final results based on the glycerol standard.

**Assessment of PG-G Hydrolysis in Intact HEK293 Cells.** HEK293 cells with or without stable overexpression of 3x hemagglutinin (3HA)-tagged hMAGL (Savinainen et al., 2010) were seeded on poly-l-lysine-coated wells of a 96-well plate and cultured in DMEM (200 µMwell) containing 10% fetal bovine serum, antibiotics, and additionally the selection antibiotic G-418 (100 µg/ml) as described earlier. After 2 days, DMEM was replaced with Hepes-buffered saline (HBS; 20 mM Hepes, pH 7.40, 140 mM NaCl, 2.5 mM MgSO4, 1 mM KCl, 1 mM CaCl2) supplemented with 0.2% (w/v) BSA and 10 mM d(+)-glucose. Cells were pretreated for 1 hour with solvent (0.1% DMSO) or the MAGL-selective inhibitor JKKK-048 (10−7 or 10−6 M final concentration) in a volume of 100 µl, after which fresh HBS containing either
solvent (0.4% ethanol) or 15d-PGJ2-G (25 µM final concentration) was added in a volume of 150 µl, followed by 120 minutes of incubation at 37°C. Aliquots (40 µl) of buffer were drawn at time points 0 (immediately after gentle plate shaking), 30, 60, and 120 minutes after substrate addition. The glycerol content was determined based on a glycerol standard.

**Verification of hMAGL-Catalyzed 15d-PGJ2-G Hydrolysis by High-Performance Liquid Chromatography.** Incubations with assay conditions mimicking the glycerol assays were performed for 90 minutes using lysates of Mock-HEK293 or hMAGL-HEK293 cells. The incubation volume was scaled up to 400 µl. At time points of 0 and 90 minutes, 100-µl aliquots were removed, acetoneitrile (200 µl) was added to stop the enzymatic reaction, and the pH of the samples was simultaneously decreased to 3.0 with phosphoric acid (H3PO4, added to acetoneitrile) to stabilize the 2-isomer against acyl migration (Saario et al., 2004). Before the high-performance liquid chromatography (HPLC) analysis, samples were centrifuged at 14,000 rpm for 4 minutes (room temperature), 150 µl of the supernatant was taken into a HPLC vial, and 30 µl of 30 mM H3PO4 was added. The samples were analyzed by the Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Karlsruhe, Germany), which consisted of a binary pump, a vacuum degasser, autosampler, and a UV detector. The separations were accomplished by a Zorbax Eclipse XDB-C18 reversed-phase column (4.6 × 50 mm, 1.8 μm; Agilent Technologies, Palo Alto, CA). The UV detector was set at 310 nm. The injection volume was 10 µl. An isocratic-mobile phase mixture of 45% phosphate buffer (30 mM, pH 3.0) in acetoneitrile was used at a flow rate of 2.0 ml/min⁻¹ at 50°C. Retention times were 2.3 minutes and 2.5 minutes for the 2- and 1 (3)-isomers of 15d-PGJ2-G, respectively, and 4.2 minutes for 15d-PGJ2.

**Activity-Based Protein Profiling.** Activity-based protein profiling (ABPP) was conducted using the serine hydrolase-targeting fluorophosphonate (FP) probe tetramethylrhodamine (TAMRA-FP) (ActivX Fluorophosphonate Probes, Thermo Fisher Scientific, Rockford, IL) as previously described elsewhere (Navia-Paldanius et al., 2012). Briefly, lysates (25 or 100 µg protein for HEK293 cells or HUVEC, respectively) were preincubated with 0.5 µl of vehicle (DMSO) or JJKK-048 (10⁻⁷ M final concentration) for 1 hour at room temperature. After this, the serine hydrolases were labeled with TAMRA-FP (2 µM final concentration) for 1 hour at room temperature. The reaction was stopped by adding a 2x SDS-loading buffer, and the proteins resolved in SDS-PAGE together with molecular weight standards. Protein bands were visualized after in-gel fluorescent gel scanning using a Fujifilm (FLA-3000; Tokyo, Japan) scanner (ex 390 nm, 570 nm emission).

**Detection of Nuclear and Cytoplasmic Nuclear Factor Erythroid 2-Related Factor.** Immunoblotting against nuclear and cytoplasmic nuclear factor erythroid 2-related factor (Nrf2) was performed essentially as previously described (Jyrkkänen et al., 2008). Briefly, after 2 hours of treatment, the cells were lysed, and the total protein concentration was measured with the BCA-assy (Pierce Biotechnology, Rockford, IL). Nuclear and cytoplasmic fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology). We used 10 to 20 µg of protein for electrophoresis. The proteins were transferred to nitrocellulose membrane, blocked, and incubated with primary antibodies. The primary antibodies used were rabbit polyclonal anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-Lamin B1 (Abcam, Cambridge, United Kingdom). Blots were visualized using horse radish peroxidase–conjugated secondary antibodies and Supersignal chemiluminescence substrate (Pierce Biotechnology). Blots were visualized using ECL Plus Western Blotting Detection System with Typhoon 9400 (GE Healthcare, Little Chalfont, United Kingdom). Protein expression data were quantified with ImageQuant TL 7.0 software (GE Healthcare).

**Nrf2 Reporter Gene Assay.** Mock-HEK293 and HKEK293 cells stably expressing 3HA-hMAGL (Savinainen et al., 2010) were seeded on 96-well plates and transfected the next day with the calcium phosphate transfection method using the following plasmids: 20 ng of pGL2-SV40 as control or pGL2-SV40-2xGCLM-ARE-luciferase (Hurtilla et al., 2008), and for normalization, with 20 ng of pCMV-β-gal vector. At 24 hours after transfection, the cells were treated with the indicated concentrations of 15d-PGJ2 or 15d-PGJ2-G in DMEM supplemented with 0.5% (w/v) BSA. At 16 hours after treatment, luciferase activities were measured using the Biotite Reporter Gene Assay (PerkinElmer Life Sciences, Waltham, MA) and Victor 1420 Multilabel Counter (Wallac, Turku, Finland). Luciferase activities were normalized to β-galactosidase activities measured as previously described elsewhere (Hurtilla et al., 2008). The results are presented as fold-change versus pGL2-SV40-control vector for each treatment.

**RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction.** HEK293 cells with or without (mock) stable 3HA-hMAGL expression were seeded on 12-well plates and treated with 15d-PGJ2 or 15d-PGJ2-G for 8 hours. The cells were collected, and the RNA was extracted with TRI Reagent (Sigma-Aldrich). The cDNA was produced with the Transcripter First Strand Synthesis Kit (Roche, Mannheim, Germany). The relative expression levels were measured according to the manufacturer’s protocol with quantitative real-time polymerase chain reaction (StepOnePlus Real-Time PCR systems; Applied Biosystems, Foster City, CA) using specific assays-on-demand (Applied Biosystems) target mixes for heme oxygenase 1 (HMOX1, Hs00157985_m1), glutamate-cysteine ligase modifier subunit (GCLM, Hs00157694_m1), and NADPH oxidoreductase 1 (NQO1, Hs00168547_m1). The expression levels were normalized to β-2-microglobulin (Hs00187842_g1) expression and are presented as fold-change in the expression versus control.

**Molecular Modeling.** Molecular modeling was performed using the Schrödinger Maestro software package (Schrödinger Suite 2013-3; Schrödinger, LLC, New York, NY). Structures of small molecules were prepared using the LigPrep module. X-ray crystal structure of the human MAGL was used (pdb:3PE6 (Schalk-Hihi et al., 2011), and the biomolecule was preprocessed using the protein preparation wizard to optimize the hydrogen bonding network and to remove any possible crystallographic artifacts (Protein Preparation Wizard; Schrödinger, LLC). Molecular docking studies were performed following the Induced Fit docking protocol. The grid box was centered using the corresponding X-ray ligand as the template, and at least one hydrogen bond was required to be formed with Ala51 or Met123. Initial docking poses were generated using Glide, where after which residues close to the ligand pose (5Å) were further refined using Prime. Redocking was performed using the SP settings of Glide. The graphical illustrations were generated using MOE software (Molecular Operating Environment, version 2013.08; Chemical Computing Group, Montreal, QC, Canada).

**Data Reproducibility and Statistical Analyses.** All numerical data are presented as mean ± S.E.M. or S.D. from at least two independent experiments, as detailed in Results. The Kₘ and Vₘₐₓ values were calculated from nonlinear regressions using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA). Statistical comparisons were performed using one-way analysis of variance, followed by Tukey's nonparametric test. Asterisks denote statistical significance (*P < 0.05, **P < 0.01, and ***P < 0.001).

**Results**

**PG-G Hydrolysis by the Human Endocannabinoid Hydrolases; hABHD6 Readily Uses PGD₂-G, and hMAGL Shows Highest Preference Toward 15d-PGJ₂-G.** Our previous study disclosed distinct MAG substrate profiles for the human serine hydrolases ABHD6, ABHD12, and MAGL (Navia-Paldanius et al., 2012). Here, we have extended this comparative substrate profiling to cover COX-2–derived oxygenation products of 2-AG by testing the utilization of four commercially available PG-G species: 15d-PGJ₂-G, PGD₂-G, PGE₂-G, and PGF₂α-G, at 25 µM concentration. The chemical
structures and calculated logP values of the PG-G species are shown in Supplemental Fig. 1. While 15d-PGJ2-G represented the 2-isomer, the other PG-G species were 1(3)-isomers. For reference purposes, we also tested in parallel 1(3)-AG, as this is the preferred endocannabinoid isomer for hABHD6 and hABHD12, whereas hMAGL uses both isomers at similar rates (Navia-Paldanius et al., 2012; Laitinen et al., 2014). To facilitate comparison with the previous MAG profiling data, we used the TEMN buffer ([50 mM Tris-HCl pH 7.40, 1 mM EDTA, 5 mM MgCl2, 100 mM NaCl], pH 7.4) supplemented with 0.5% BSA (w/v), as this buffer system has proven optimal for studies exploring 2-AG–evoked cannabinoid receptor signaling and endocannabinoid degradation in cellular lysates and native membrane preparations (Savinainen et al., 2003; Saario et al., 2004; Navia-Paldanius et al., 2012; Laitinen et al., 2014).

In accordance with our preliminary findings with the ability of 3HA-hMAGL (short isoform) to hydrolyze PG-G species (Laitinen et al., 2014), untagged hMAGL (long isoform) readily hydrolyzed all tested PG-G species, and notably at 2- to 3-fold higher rates as compared with 1-AG (Fig. 1A). The best substrate in this series was 15d-PGJ2-G. Also, it is notable that the hydrolysis rates of 15d-PGJ2-G rivaled those of the best MAG species, previously determined under identical assay conditions (Navia-Paldanius et al., 2012). For hMAGL, the relative hydrolysis rate for the tested substrates was 15d-PGJ2-G > PGD2-G > PGF2α-G > PGE2-G > 1-AG.

Although the MAG substrate profiles of hMAGL and hABHD6 were previously shown to be closely related (Navia-Paldanius et al., 2012), the utilization of PG-G species was a clear deviation from this. As shown in Fig. 1B, hABHD6 readily hydrolyzed PGD2-G at rates corresponding to ~60% of those for 1-AG. In contrast, the hydrolysis rates of the other PG-G species were modest, being ~15–30% of those measured for 1-AG. For hABHD6, the relative hydrolysis rate was 1-AG > PGD2-G > 15d-PGJ2-G > PGF2α-G ≥ PGE2-G.

For hABHD12, the preferred MAG substrate was 1(3)-AG, and it sparingly used other tested MAG species (Navia-Paldanius et al., 2012). Here, we found that the hABHD12-dependent hydrolysis rates of the PG-G species were modest, being merely ~10–20% of those determined for 1-AG (Fig. 1C). For hABHD12, the relative hydrolysis rate was 1-AG > > PGE2-G > PGD2-G ≥ PFG2α-G ≥ 15d-PGJ2-G.

As previously demonstrated (Navia-Paldanius et al., 2012), the high sensitivity of the fluorescent glycerol assay afforded hydrolase activity measurements with only 0.3 µg of lysate protein per well, ensuring that background cellular activity was negligible also with the current set of substrates (Fig. 1D). As PG-G hydrolysis was particularly robust for MAGL, we focused rest of these studies to the characterization of this response in more detail.

**Verification of hMAGL-Catalyzed 15d-PGJ2-G Hydrolysis by HPLC.** Hydrolysis of 15d-PGJ2-G yields equimolar quantities of 15d-PGJ2 and glycerol as the end products (Fig. 2A). To complement the results of the glycerol assay, an HPLC-based method to detect 15d-PGJ2 was employed. The HPLC analysis revealed that the substrate eluted as two peaks: the major peak (~90%) representing the 2-isomer and the minor peak (~10%), representing the 1(3)-isomer (Fig. 2B). Given that nonenzymatic acyl migration is expected to take place in aqueous milieu (Saario et al., 2004), incubating 15d-PGJ2-G together with

![Fig. 1.](molpharm.aspetjournals.org)
Mock-HEK lysates for 90 minutes resulted in a notable change in the 1(3)-to-2-isomer ratio, and importantly, no detectable amount of 15d-PGJ₂ was formed (Fig. 2C). In parallel incubations together with hMAGL-HEK lysates, a clear peak was observed, eluting at the position of 15d-PGJ₂ standard, and with a concomitant decrease in the peak corresponding to 15d-PGJ₂-G (Fig. 2D). Quantification based on the peak area corresponding to 15d-PGJ₂ from three independent experiments indicated that while 15d-PGJ₂ was undetectable in all experiments with Mock-HEK lysates, parallel incubations with hMAGL-HEK

Fig. 2. HPLC verification of hMAGL-catalyzed 15d-PGJ₂-G hydrolysis. (A) Reaction scheme for 15d-PGJ₂-G hydrolysis generating equimolar quantities of 15d-PGJ₂ and glycerol. (B) Chromatographs showing the elution pattern of 15d-PGJ₂-G and 15d-PGJ₂ (both from Cayman Chemicals). Note that the substrate is a mixture of the 2- and 1(3)-isomers (∼90 and ∼10%, respectively). (C) HPLC analysis of the 15d-PGJ₂-G metabolites after incubation for 90 minutes with Mock-HEK293 lysates. Note the change in the ratio of the substrate isomers due to nonenzymatic acyl migration in an aqueous milieu. Note also the absence of any product eluting at the position of 15d-PGJ₂. (D) HPLC analysis of the 15d-PGJ₂-G metabolites after incubation for 90 minutes with hMAGL-HEK293 lysates. Note the presence of a new peak eluting at the position of 15d-PGJ₂. Data are from one experiment that was repeated twice. Quantification based on the peak area corresponding to 15d-PGJ₂ from three independent experiments indicated that although 15d-PGJ₂ was below the detection limit in incubations using Mock-HEK293 lysates, parallel incubations with hMAGL-HEK293 lysates yielded 1.5 ± 0.1 nmol of 15d-PGJ₂ (mean ± S.E.M., n = 3).
lysates yielded 1.5 ± 0.1 nmol of 15d-PGJ2 (mean ± S.E.M., n = 3), a value closely matching the estimated amount of glycerol formed at the time point 90 minutes, extrapolated from the reaction rate at time points 0–30 minutes of the kinetic fluorescent assay. Thus, the HPLC analysis validated results of the glycerol assay showing that MAGL-catalyzed 15d-PGJ2-G hydrolysis generates the two expected end products.

**MAGL Splice Variants Use 15d-PGJ2-G at Similar Rates.** Based on molecular and Western blotting evidence, MAGL is expressed in a tissue-specific manner, usually migrating as a single ~33 kDa protein band or as a ~33–35 kDa doublet, depending on the tissue (Karlsson et al., 2001). Two MAGL isoforms are reported in the GenBank (long isoform 1; 313 amino acids, accession code NP-001003794 and short isoform 2; 303 amino acids, accession code NP-009214) (Labar et al., 2010). The findings presented here were obtained using the long isoform (hMAGL-long). It is not currently known why different MAGL isoforms exist or whether they display distinct catalytic properties and/or substrate preferences. To unveil possible differences in substrate preferences, we transiently expressed the human and mouse short MAGL isoforms (hMAGL-short and mMAGL-short, respectively) in HEK293 cells and visualized the SDS-PAGE mobility pattern of catalytically active isoforms using ABPP with the active site serine-targeting probe TAMRA-FP. As previously reported (Navia-Paldanius et al., 2012), hMAGL-long migrated mainly as a ~33–35 kDa doublet (Fig. 3A), whereas the short isoform migrated as a single band of ~33 kDa (human) or ~34 kDa (mouse). The crude substrate profiling of MAGL isoforms was performed using 2-AG, 15d-PGJ2-G and the preferred MAG substrate 1-decanoyl-rac-glycerol (1-DG) (Navia-Paldanius et al., 2012), all tested at 5 and 25 μM final concentrations. These experiments revealed that the MAGL splice variants shared similar substrate profile in that 1-DG was used at the highest rates, and all isoforms showed a clear preference for 15d-PGJ2-G over 2-AG (Fig. 3, B–D).

**Substrate Availability for hMAGL-Catalyzed Hydrolysis in Assays with or without BSA.** The experiments described so far were all conducted using our routine assay protocol with BSA (0.5% w/v) included as a carrier for lipophilic compounds. Using the routine protocol, we determined the $K_m$ and $V_{max}$ values for hMAGL (long isoform) toward 15d-PGJ2-G covering the concentration range 3–50 μM. Due to robust expression of the hMAGL-gene construct in HEK293 cells, the lysates were tested at two dilutions (1:10 and 1:20, both containing 0.3 mg protein/well, diluted with HEK lysates) to ensure that substrate availability was not a limiting factor and that the activity measurements were within the linear range of the glycerol standards (Navia-Paldanius et al., 2012). Nonlinear fitting of the substrate-velocity data indicated that the $K_m$
values for 15d-PGJ2-G were similar (12–17 μM) regardless of the enzyme dilution (Fig. 4, A and B). For the enzyme dilutions 1:20 and 1:10, the maximal velocities ($V_{\text{max}}$) were 38 ± 2 and 75 ± 5 nmol/mg per minute, respectively.

We were curious to learn whether omission of BSA might affect substrate availability, so we compared the use of selected substrates (2-AG, 1-DG, and 15d-PGJ2-G) for MAGL-catalyzed hydrolysis in incubations with or without BSA. The substrates were tested at 25 μM final concentrations with the outcome that while the hydrolysis rates of 15d-PGJ2-G were comparable under both conditions, those of 2-AG were significantly higher and those of 1-DG significantly lower in incubations without BSA (Fig. 4C). Using Michaelis-Menten kinetics, we determined the $K_m$ values for hMAGL-catalyzed hydrolysis of 2-AG, 15d-PGJ2-G, 1-DG, and PGD2-G in incubations without BSA. Under such conditions, the $K_m$ values were 9.7 ± 3.6, 12.2 ± 4.6, and 20.0 ± 2.6 μM for 2-AG, 15d-PGJ2-G, and 1-DG, respectively (Fig. 4D). Noteworthy, hardly any hydrolysis of PGD2-G was detected in the absence of BSA, precluding accurate $K_m$ determinations (Fig. 4D).

**Molecular Modeling Highlights Key Interactions of PG-G Species with the MAGL Active Site.** We considered various possibilities to explain why 15d-PGJ2-G was the preferred MAGL substrate in the PG-G series. The findings herein indicated that substrate availability might be one determinant. Moreover, it should be noted that 15d-PGJ2-G represented the 2-isomer, while the other PG-G species were 1(3)-isomers. It is known from previous studies that MAGL hydrolyses 1(3)- and 2-monoacylglycerols with similar rates (Saario et al., 2004; Navia-Paldanius et al., 2012; Laitinen et al., 2014). Unfortunately, restricted availability of additional PG-G species precluded further studies that would directly compare utilization of PG-G isomer pairs.

To gain molecular level insights into this issue, molecular modeling studies were employed. These studies suggested that the PG-G species all converged well to the MAGL active site regardless of the isomer; we found no major differences in the docking of the glycerol moiety. However, the prostaglandin moiety in 15d-PGJ2-G offered less hydrophilic interactions with the MAGL residues as compared with the other PG-G species (Fig. 5). Interestingly, the additional hydrogen bond contacts of

![Fig. 4. Substrate availability for hMAGL-catalyzed hydrolysis in assays with or without BSA. (A) Michaelis-Menten kinetics for hMAGL-catalyzed hydrolysis of 15d-PGJ2-G under routine assay conditions containing 0.5% BSA (w/v). To ensure activity measurements within tolerable substrate consumption and the linear range of glycerol assay, hMAGL-HEK lysates were diluted 1:10 or 1:20 with Mock-HEK lysate. Lysates (0.3 μg/well) were incubated with the indicated concentrations of 15d-PGJ2-G, and the $K_m$ and $V_{\text{max}}$ values were determined at the time point of 30 minutes where substrate availability was not rate limiting. (B) The $K_m$ and $V_{\text{max}}$ values were calculated as nonlinear regressions using GraphPad Prism 5.0 for Windows. The lines represent Lineweaver-Burk plots of the data in A. Values are mean ± S.D. from two independent experiments. (C) Substrate availability for MAGL-catalyzed hydrolysis in incubation with (+) or without (−) BSA. Lysates (0.3 μg/well) of HEK293 cells transiently overexpressing hMAGL were incubated together with the indicated substrates (25 μM final concentration) in the presence or absence of 0.5% BSA. Glycerol output was determined at the time point of 30 minutes where substrate availability was not rate limiting. The $K_m$ and $V_{\text{max}}$ values were calculated as nonlinear regressions using GraphPad Prism 5.0 for Windows and were 9.7 ± 3.6, 12.2 ± 4.6, and 20.0 ± 2.6 μM for 2-AG, 15d-PGJ2-G, and 1-DG, respectively. Due to negligible hydrolysis, no accurate $K_m$ value could be determined for PGD2-G. Values are mean ± S.E.M. from three to four independent experiments except for PGD2-G where n = 2.

![Fig. 5. Molecular modeling highlights key interactions of PG-G species with the MAGL active site.](image-url)
the other PG-G species were located to the lid area at the active site entry (Supplemental Fig. 2). Theoretically, such contacts may retard the release of the prostaglandin products, potentially slowing down hydrolysis rates of the 1(3)-isomers.

It is also worth noticing that the 1(3)-isomers possess a chiral carbon, indicating that they represent a racemic mixture of 1-G(R) and 1-G(S) forms. Docking the R and S forms individually to the MAGL active site gave practically similar hydrogen bond interactions with the key active site residues Met123, Tyr194, Ser122, Ala51, and His121 (Fig. 5, B–D). In the case of 15d-PGJ2-G, although contacts to Met123, Ser122, Ala51, and His121 were well preserved, hydrogen bonding to Tyr194 was not always present (Fig. 5A).

Collectively, these observations suggest that the chiral and flexible 1(3)-PG-G isomers are able to form a set of similar hydrogen bond interactions in various orientations with active site residues Tyr194, Ser122, and His121, in accordance with earlier findings concerning MAG substrates (Bertrand et al., 2010; Laitinen et al., 2014). The importance of Tyr194 was highlighted in our previous study (Laitinen et al., 2014) where mutation of this residue was shown to affect equally hydrolysis rates of 2- and 1(3)-MAG species, while mutation of Cys242 disrupted the balanced hydrolysis of the isomers in favor of the 2-isomers. These findings could at least partly explain why 15d-PGJ2-G without the chiral center was hydrolyzed at higher rates as compared with the other tested PG-G species.

**Activation of Nrf2 Signaling Pathway;** Both 15d-PGJ2 and 15d-PGJ2-G stimulate nuclear translocation of Nrf2 in HUVEC endogenously expressing MAGL. Previous studies have shown that electrophilic compounds such as 15d-PGJ2 can signal via several pathways, including Nrf2 and peroxisome proliferator-activated receptor-γ (PPAR-γ) mediated pathways, to evoke cellular responses (Strauss and Glass 2001; Kansanen et al., 2009, 2012; Harmon et al., 2011; Haskew-Layton et al., 2013). As 15d-PGJ2 and 15d-PGJ2-G possess two highly electrophilic α,β-unsaturated carbonyls, both compounds could engage similar signaling functions. Whether 15d-PGJ2-G is capable of mediating such functions is not yet known, although recent studies have implicated that 15d-PGJ2-G may act as a PPAR-γ activating ligand, capable of suppressing nuclear factor of activated T cells (NFAT) transcriptional activity and interleukin-2 (IL-2) expression in activated T cells (Raman et al., 2011, 2012).

However, time-dependent hydrolysis of 15d-PGJ2-G to 15d-PGJ2 likely complicates straightforward conclusions that could be drawn from studies requiring prolonged incubation times for a functional readout. To address this issue, we used HUVEC, which have shown to be a useful cellular model in studies exploring electrophile signaling (Jyrkkänen et al., 2017).

**Fig. 5.** Docking of the studied PG-G species into the MAGL active site. Selected docking poses of (A) 15d-PGJ2-G (green), (B) PGD2-G (orange), (C) PGE2-G (magenta), and PGF2a-G (cyan) to the MAGL crystal structure (pdb:3PE6) illuminating the key hydrogen bonding interaction of favorable docking poses. Catalytic triad residues (Ser122-Asp239-His269) are colored using yellow carbons, and the residues forming the oxyanion hole (Met123 and Ala51) are also illustrated. Selected substrate specific hydrogen bond forming residues are also shown. In the case of the PGD2-G (B), 1(3)-glycerol moiety is in S-configuration, whereas panels (C) and (D) represent the R-isomers of PGE2-G and PGF2a-G, respectively.
2008; Kansanen et al., 2009). Competitive ABPP revealed that HUVEC endogenously express MAGL, as evidenced by the presence of a JJKK-048-sensitive protein doublet migrating at the 33–35 kDa range in SDS-PAGE (Fig. 6A). JJKK-048 is a highly potent MAGL-selective inhibitor (Aaltonen et al., 2013). Hydrolase assays indicated that MAGL fully accounted for 15d-PGJ2-G hydrolysis in HUVEC lysates, as this activity was potently (IC50 ~0.2 nM) and comprehensively blocked by JJKK-048 (Fig. 6B).

Using intact HUVEC, we compared the capacity of 15d-PGJ2 and 15d-PGJ2-G to induce nuclear enrichment of Nrf2, an early measurable step in Nrf2 signal transduction (Fig. 6C). Our previous studies have shown that Nrf2 translocation is detectable in HUVEC after 2-hour stimulation with oxidized phospholipids (Jyrkkänen et al., 2008). To achieve optimal signal and to avoid prolonged incubation times, we chose the 2-hour time point for closer inspection. The results indicated that when tested at 5 μM concentration 15d-PGJ2 and 15d-PGJ2-G to the same extent evoked nuclear enrichment of Nrf2 in the endothelial cells. Of note, the MAGL-selective inhibitor JJKK-048 had no effect on this response (Fig. 6D). Thus, in HUVEC with endogenous MAGL expression, the electrophiles similarly induced nuclear translocation of Nrf2, and this response was independent of MAGL activity.

15d-PGJ2-G Hydrolysis in Intact HEK293 Cells. We assessed 15d-PGJ2-G hydrolysis in situ using HEK293 cells with or without stable 3HA-hMAGL expression (Savinainen et al., 2010). Cells were pretreated for 1 hour with solvent (DMSO or the MAGL-inhibitor JJKK-048) and incubated thereafter for further 2 hours in HBS with or without 15d-PGJ2-G (25 μM final concentration). The buffer was sampled at various time points, and the amount of liberated glycerol was determined.

Fig. 6. 15d-PGJ2 and 15d-PGJ2-G both stimulate nuclear translocation of Nrf2 in HUVEC endogenously expressing MAGL. (A) Competitive APBB of HUVEC lysates unveiling endogenous expression of catalytically active serine hydrolases, including MAGL (a protein doublet migrating at 33–35 kDa whose labeling by the active-site directed probe is prevented by the MAGL-selective inhibitor JJKK-048 (100 nM). The image is representative from three separate ABPP runs with a similar outcome (B). MAGL fully accounts for 15d-PGJ2-G hydrolysis in HUVEC lysates, as evidenced by comprehensive blockade of this hydrolysis by the MAGL-selective inhibitor JJKK-048 (IC50 178 pM) and by the fact that JJKK-048 inhibits 15d-PGJ2-G hydrolysis to the same extent as seen with the globally acting serine hydrolase inhibitor methylarachidonoyl fluorophosphonate (MAFP) (10^-4 M). (C) Schematics and temporal aspects of stress-activated Nrf2 signaling pathway. Under basal conditions, Nrf2 is targeted for proteosomal degradation. Electrophilic or oxidative stress leads to Nrf2 translocation into the nucleus to increase transcription of target genes such as heme oxygenase 1 (HMOX1), glutamate-cysteine ligase modifier subunit (GCLM), and NAD(P)H quinone oxidoreductase-1 (NQO1), all containing the antioxidant response element (ARE) in the 5’-regulatory region. (D) 15d-PGJ2 and 15d-PGJ2-G stimulate nuclear translocation of Nrf2 in HUVEC equally well. The MAGL inhibitor JJKK-048 (2 x 10^-7 M) has no effect on this response. Bottom: representative Western blots of the nuclear Nrf2 signal in comparison with lamin B1 that was used as a loading control. In (B), the data are mean ± S.E.M. of duplicate wells from two independent experiments. In (D), the data are mean ± S.E.M. from three independent experiments. Statistical comparisons were done using one-way analysis of variance, followed by Tukey’s multiple comparisons, and statistical significance is indicated with asterisks (***P < 0.001).
indicated that significant 15d-PGJ$_2$-G hydrolysis was evident in Mock cells only after 60 minutes, whereas in 3HA-hMAGL-HEK293 cells the substrate was efficiently hydrolyzed already at the earliest time point examined (Fig. 7). As expected, 15d-PGJ$_2$-G hydrolysis in hMAGL-HEK293 cells was sensitive to JJKK-048, whereas this inhibitor had no effect on 15d-PGJ$_2$-G hydrolysis in Mock-cells.

Collectively, these experiments revealed relatively low endogenous hydrolytic activity toward 15d-PGJ$_2$-G in HEK293 cells, whereas with hMAGL expression, 15d-PGJ$_2$-G hydrolysis was clearly accelerated. We next wished to systematically compare the ability of 15d-PGJ$_2$ and 15d-PGJ$_2$-G to activate the Nrf2 signaling pathway, and we used multiple readouts for Nrf2 activation, including nuclear localization, luciferase reporter assays, and the ability of the electrophiles to set in train Nrf2 target gene expression.

**15d-PGJ$_2$ and 15d-PGJ$_2$-G Similarly Activate Nrf2 Signaling Pathway and Its Target Gene Expression in HEK293 Cells with or without hMAGL Overexpression.**

As in the studies with HUVEC, we used Western blot analysis to monitor nuclear enrichment of Nrf2 after 2-hour stimulation with 15d-PGJ$_2$ or 15d-PGJ$_2$-G (5 μM final concentration) (Fig. 8A). The outcome was similar in cells with or without 3HA-hMAGL expression, although a nonsignificant trend toward potentiation of the 15d-PGJ$_2$-G response in 3HA-hMAGL-HEK cells treated with the MAGL-inhibitor JJKK-048 was evident. Dose-response studies of electrophile-driven antioxidant response element (ARE) activation revealed that 15d-PGJ$_2$-G stimulated this response significantly better as compared with 15d-PGJ$_2$ (Fig. 8B). Again, the outcome was similar regardless of 3HA-hMAGL expression.

As a distal step in Nrf2 signaling, induction of three Nrf2 target genes (HMOX1, GCLM, and NQO1) was examined in response to electrophile stimulation (0–5 μM). These results indicated that 15d-PGJ$_2$-G induced HMOX1 mRNA levels significantly more efficiently than did 15d-PGJ$_2$ (Fig. 8C). A similar, albeit nonsignificant, trend was also evident for the induction of GCLM and NQO1 (Fig. 8, D and E). The observed responses were similar between Mock-HEK and 3HA-hMAGL-HEK cells, the sole exception being that no statistically significant increase in NQO1 transcripts was detected in 3HA-hMAGL-HEK cells (Fig. 8, C–E).

### Discussion

In this study, we have demonstrated that the human endocannabinoid hydrolases MAGL, ABHD6, and ABHD12 can variably hydrolyze PG-G species in vitro and that hMAGL robustly hydrolyzed all tested PG-G species, and at rates rivaling those of the best MAG substrates evaluated under comparable conditions. We demonstrated that hMAGL showed particular preference toward 15d-PGJ$_2$-G. Michaelis-Menten kinetics revealed that hMAGL avidly (K$_{m}$ ~15 μM) bound 15d-PGJ$_2$-G. Whereas the availability of 15d-PGJ$_2$-G for MAGL-catalyzed hydrolysis was similar with or without BSA, the importance of the protein carrier in delivery of PGD$_2$-G for enzymatic hydrolysis was demonstrated. Molecular docking studies provided atomic level insights into variable interactions of the PG-G isomers with the MAGL active site. We demonstrated that the MAGL isoforms shared a similar substrate profile, and all robustly hydrolyzed 15d-PGJ$_2$-G. We showed that hMAGL used 15d-PGJ$_2$-G also in living HEK293 cells engineered to express this enzyme. In HUVEC lysates, endogenous MAGL fully accounted for 15d-PGJ$_2$-G hydrolysis. To explore the consequences of this activity, we assessed the
ability of 15d-PGJ2-G to activate the canonical Nrf2 signaling pathway known to be used by the hydrolysis end product 15d-PGJ2, and these studies unveiled for the first time that 15d-PGJ2 and 15d-PGJ2-G both activated the Nrf2 pathway as well as transcription of several target genes of this pathway. Our findings challenge previous claims regarding the ability of MAGL to use PG-G species and extend the MAGL substrate profile beyond the classic MAG species.

MAGL Acts as a PG-G Hydrolase In Vitro; BSA Variably Affects Substrate Availability

Previously, the ability of PGD2-G, PGE2-G, and PGF2a-G to inhibit 2-oleoylglycerol (2-OG) hydrolysis by rat brain cytosolic MAGL preparation was examined (Fowler and Tiger 2005). This study indicated that the tested PG species were weak inhibitors of 2-oleoylglycerol hydrolysis, suggesting that these compounds were not efficiently competing with the substrate. On the other hand, 15d-PGJ2 at the low-micromolar range (IC50 ~50 μM) was reported to inhibit hMAGL-mediated 4-nitrophenylacetate hydrolysis (Björklund et al., 2010). In light of the present findings, this could represent end-product inhibition of MAGL activity. We tested this possibility by using a competitive approach where MAGL-catalyzed hydrolysis of 15d-PGJ2-G and 1-AG was evaluated in the presence of increasing concentrations of 15d-PGJ2. We found, however, that the end product did not affect the hydrolysis rates of these substrates, not even when present at 8-fold excess quantities (Supplemental Fig. 3).

Fig. 8. Comparative analysis of activation of Nrf2 signaling pathway by 15d-PGJ2 and 15d-PGJ2-G in HEK293 cells with or without hMAGL overexpression. (A) Nuclear translocation of Nrf2 in response to 5 μM 15d-PGJ2 and 15d-PGJ2-G in Mock-transfected (HEK) and hMAGL-HEK cells. Note the weak response to 15d-PGJ2 in HEK293 cells as compared with HUVEC (Fig. 6). Note also that although one-way analysis of variance indicated no statistically significant differences between the treatments, the MAGL inhibitor JKKK-048 (0.2 μM) shows a clear trend in amplifying the 15d-PGJ2-G response in hMAGL-HEK cells. Bottom: representative Western blots of the nuclear Nrf2 signal in comparison with the lamin B1 that was used as a loading control. (B) Dose-dependent antioxidant response element (ARE) activation by 15d-PGJ2 and 15d-PGJ2-G in HEK293 cells (left) and hMAGL-HEK cells (right). Dose-dependent average induction of antioxidants of the ARE target genes heme oxygenase 1 (HMOX1) (C), glutamate-cysteine ligase modifier subunit (GCLM) (D), and NAD(P)H quinone oxidoreductase-1 (NQO1) (E) by 15d-PGJ2 and 15d-PGJ2-G in HEK293 cells with or without hMAGL overexpression. Data are mean ± S.E.M. from triplicate wells from a representative experiment that was repeated once with a similar outcome. Statistical comparisons were performed using one-way analysis of variance, followed by Tukey’s multiple comparisons, and statistical significance is indicated with asterisks (∗P < 0.05; **P < 0.01; ***P < 0.001) or hashtags (#P < 0.05; ###P < 0.001).
Very few studies have directly assessed the ability of MAGL to use the PG-G species. The first study (Vila et al., 2007), using purified rat MAGL and PGE₂-G and PGF₂α-G as the substrates, concluded that in comparison with 2-AG the PG-G species were poor MAGL substrates, a view that prevails in the literature (Rouzer and Marnett, 2011). More recently and quite the opposite, Xie et al. (2010) reported that hMAGL efficiently hydrolyzed PGE₂-G and PGF₂α-G. In line with these results, we found that 3HA-hMAGL readily used various PG-G species (Laitinen et al., 2014). Our present work not only confirms but also substantially extends these preliminary findings.

We demonstrated that restricted substrate availability due to absence of protein carrier was a critical factor constraining successful detection of MAGL-catalyzed PGD₂-G hydrolysis. This finding could potentially also explain the negative outcome of the original study (Vila et al., 2007). In contrast to the methodology used in that study, our assays as well as those used by Xie et al. (2010) contained BSA as a carrier for lipophilic substrates. We argue that inclusion of BSA is fully justified, clearly facilitating availability of substrates like 1-DG for enzymatic hydrolysis.

On the other hand, the carrier may avidly bind other substrates (as illustrated here for 2-AG), restricting thereby the availability for enzymatic hydrolysis. We noted that availability of 2-AG was similarly affected by bovine and human albumin (0.5%) whereas 0.05% albumin behaved like the aqueous buffer alone (Supplemental Fig. 4). However, tight binding to BSA does not totally occlude substrate availability but is typically reflected as lowered affinity toward the enzyme (i.e., higher \( K_m \) values). In the case of 2-AG, \( K_m \) values around 100–200 \( \mu M \) were obtained for MAGL-catalyzed hydrolysis in assays containing BSA (Navia-Paldanias et al., 2012; Laitinen et al., 2014), whereas a \( K_m \) value of \( \sim 10 \) \( \mu M \) was determined here in incubations without BSA (Fig. 4D). Yet for other substrates such as 15d-PGJ₂-G, hydrolysis rates (and \( K_m \) values) were comparable in the presence and absence of the carrier. It is noteworthy that the robust hydrolysis of 2-AG as opposed to the barely detectable hydrolysis of PGD₂-G in our assays without BSA is reminiscent of the findings of the original study with the negative outcome (Vila et al., 2007).

Molecular modeling further supported favorable interaction of the PG-G species with the hMGL active site. These studies suggested that due to lack of chiral carbonyl center, 15d-PGJ₂-G (2-isomer) was more favorably posed to the MAGL active site as compared with the 1(3)-isomers possessing the chiral center. Modeling studies indicated further that in contrast with the other PG-G species, 15d-PGJ₂-G possessing the most lipophilic prostaglandin tail did not form efficient hydrogen bond contacts within the lid area of MAGL. Theoretically, such contacts could retard the release of the prostaglandin products, potentially slowing down hydrolysis rates of the 1(3)-isomers. This line of reasoning is consistent with previous observations suggesting that the functionality of hMAGL includes movement of the flexible lid loop between open and closed conformations upon binding of the substrate (Karageorgos et al., 2010; 2013).

15d-PGJ₂-G Hydrolysis Generates the Anti-Inflammatory Lipid 15d-PGJ₂

Cyclopentenone prostaglandins possess highly electrophilic \( \alpha, \beta \)-unsaturated carbonyls that can react with cysteine thiols, either on glutathione or cellular proteins (Buczynski et al., 2009; Kansanen et al., 2009). In fact, 15d-PGJ₂ possesses two such electrophilic centers and is considered as an endogenous anti-inflammatory lipid with a wide spectrum of physiologic activities (Scher and Pillinger 2005; Buczynski et al., 2009). For inflammation, 15d-PGJ₂ plays a role in its resolution (Rajakariar et al., 2007), and it has been identified as a high-affinity activator of PPARγ and Nrf2 signaling pathways (Buczynski et al., 2009; Kansanen et al., 2009). Using various readouts to monitor activation of the Nrf2 signaling pathway, we demonstrated that in HUVEC endogenously expressing MAGL, 15d-PGJ₂ and 15d-PGJ₂-G both increased nuclear content of Nrf2 to the same extent, and pharmacologic MAGL inhibition had no effect on this response. In HEK293 cells with or without MAGL expression, both electrophiles stimulated Nrf2 nuclear accumulation, whereas comparative dose-response studies indicated that on a molar basis 15d-PGJ₂-G was a stronger antioxidant response element (ARE) activator and inducer of Nrf2 target gene expression compared with 15d-PGJ₂. As far as we are aware, this is the first study to document that a PG-G is capable of activating the Nrf2 signaling pathway, a property previously attributed to 15d-PGJ₂.

The signaling functions of the PG-G species are currently poorly understood, but 15d-PGJ₂-G has been shown to inhibit interleukin-2 expression via PPARγ (Raman et al., 2011), and PGD₂-G has anti-inflammatory properties in macrophages via unknown receptor(s) (Alhouayeek et al., 2013). These results combined with ours imply that COX-2-derived PG-G species have potent signaling capabilities that may be modified by PG-G hydrolytic enzymes such as MAGL and ABHD6.

Possible Implications and Relevance of Enzymatic PG-G Hydrolysis

The lipid signaling pathways are closely intertwined (Kukkonen 2011; Mulvihill and Nomura 2013). In the central nervous system, 2-AG acts as a lipid mediator and its enzymatic hydrolysis not only terminates endocannabinoid signaling but concomitantly feeds the key substrate for COX-2—catalyzed production of prostaglandins, a series of lipid mediators that play crucial roles in a variety of biologic responses (Hernanson et al., 2014). Furthermore, COX-2-mediated oxygenation is emerging as the third major metabolic pathway for the endocannabinoids with potential therapeutic implications (Rouzer and Marnett 2011; Hernanson et al., 2014). Under specific conditions, 2-AG-derived PG-G species are formed by COX-2 action in vitro and in vivo, and these metabolites likely have bioactivities of their own, mediated via as-yet-unidentified non-PG receptors (Rouzer and Marnett 2011; Hernanson et al., 2014). Thus, MAGL-dependent PG-G hydrolysis, if proven relevant in vivo, could be envisioned to regulate the postulated signaling functions of PG-G species. Indeed, the first in vivo evidence supporting this possibility was recently reported (Shimizu et al., 2014).

We found that PGD₂-G was the preferred PG-G substrate for hABHD6, with hydrolysis rates ~60% of those observed for 1-AG. However, this in vitro finding does not necessarily mean that ABHD6 would use this substrate in vivo. Nevertheless, this result could be relevant in light of recent findings demonstrating that pharmacologic inhibition of ABHD6 in lipopolysaccharide-induced murine macrophages led to 2-AG accumulation and channeling to COX-2-derived anti-inflammatory PG-G species, including PGD₂-G, a precursor of 15d-PGJ₂-G.
(Alhouayek et al., 2013). Furthermore, in vivo administration of PGD₂-G was shown to reduce lipopolysaccharide-induced inflammation, confirming the biologic relevance of this 2-AG metabolite. In light of our findings, ABHD6 inhibitors might not only funnel accumulating 2-AG toward PGD₂-G generation, but could also concomitantly prolong its biologic half-life by preventing its breakdown. This should be a viable hypothesis to be tested in future in vivo studies.

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