1-1-2009

Inhibition Of Human Carboxylesterases: Exploring Interindividual Variation Of Biochemical Activity And Novels Physiological Functions Of Carboxylesterases

Shuqi Xie

INHIBITION OF HUMAN CARBOXYLESTERASES: EXPLORING INTERINDIVIDUAL VARIATION OF BIOCHEMICAL ACTIVITY AND NOVEL PHYSIOLOGICAL FUNCTIONS OF CARBOXYLESTERASES

By

Shuqi Xie

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Sciences
in the Department of Basic Sciences

Mississippi State, Mississippi

December 2009

INHIBITION OF HUMAN CARBOXYLESTERASES: EXPLORING INTERINDIVIDUAL VARIATION OF BIOCHEMICAL ACTIVITY AND NOVELS PHYSIOLOGICAL FUNCTIONS OF CARBOXYLESTERASES

By

Shuqi Xie

Approved:

Matt K. Ross Assistant Professor of Veterinary Medical Sciences (Major Professor)	Janice E. Chambers Professor of Veterinary Medical Sciences (Committee Member)
Allen Crow Assistant Research Professor of Veterinary Medical Sciences (Committee Member)	
Larry Hanson Professor of Veterinary Medical Science (Graduate Coordinator)	Kent H. Hoblet Dean of the College of Veterinary Medicine

Name: Shuqi Xie

Date of Degree: December 11, 2009

Institution: Mississippi State University

Major Field: Veterinary Medical Science

Major Professor: Dr. Matt K. Ross

Title of Study: INHIBITION OF HUMAN CARBOXYLESTERASES: EXPLORING INTERINDIVIDUAL VARIATION OF BIOCHEMICAL ACTIVITY AND NOVEL PHYSIOLOGICAL FUNCTIONS OF CARBOXYLESTERASES

Pages in Study: 68

Candidate for Degree of Master of Science

Carboxylesterases (CEs) are nonspecific hydrolytic enzymes and responsible for the metabolism of xenobiotics and endobiotics that contain ester bonds. There are two human CE isoforms found in liver, CES1 and CES2. In this study it is shown that the mere abundance of CES1 protein expression in human liver does not predict its biochemical activity. The human interindividual variation in CES1 activities may attribute to several mechanisms. One possibility is the presence of endogenous inhibitors in liver, arachidonic acid (AA) and 27-hydroxycholesterol (27-HC). CES1 is also expressed in human monocytes/macrophages and is proposed to catalyze the rate-limiting step of cholesterol ester mobilization in macrophages. It is of interest to determine whether CES1 can degrade the lipid mediators, 2-arachidonoylglycerol (2-AG), prostaglandin E_2-1-glyceryl ester (PGE_2-G), and prostaglandin $F_{2\alpha}$-1-glyceryl

ester ($PGF_{2\alpha}$-G), in monocytes/macrophages and to determine if this metabolism is inhibited by organophosphate pesticide exposure.

DEDICATION

This work is dedicated to my parents, Lixia Zhu and Jijie Xie. Thank you for your understanding and encouragement all the time.

ACKNOWLEDGEMENTS

I would like to extend a deep appreciation to my major advisor Dr. Matt Ross for his guidance during my graduate education. I also thank my committee members Dr. Allen Crow and Dr. Jan Chambers, for their support and assistance with both my education and the preparation of this thesis. Last, but not least, I would like to thank Abdolsamad Borazjani and Katye Herring for their assistance on this project.

TABLE OF CONTENTS

LIST OF SCHEMES

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CE, carboxylesterase; *p*NPV, *para*-nitrophenyl valerate; PO, paraoxon; CPO, chlorpyrifos oxon; PAGE, polyacrylamide gel electrophoresis; DMSO, di-methyl sulfoxide; AA, arachidonic acid; 27-HC, 27-Hydroxycholesterol; 22(*R*)-HC, 22(*R*)-Hydroxycholesterol; 2-AG, 2-arachidonoylglycerol; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGE_2-G, prostaglandin E_2-1-glyceryl ester; $PGF_{2\alpha}$-G, prostaglandin $F_{2\alpha}$-1-glyceryl ester; PG-Gs or PG-G, prostaglandin glyceryl esters; PGs, prostaglandins; MAGL, monoacylglycerol lipase; FAAH, fatty acid amide hydrolase; COX-2, cyclooxygenase-2; AEA, anandamide; FP-biotin, fluorophos-phonate-biotin; HPETE, hydroperoxyeicosatetraenoic acid; EETs, epoxyeico-satetraenoic acids; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; NAM, *N*-arachidonoyl maleimide; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; ACN, acetonitrile; ATCC, American Type Culture Collection; PTMs, post-translational modifications

CHAPTER I

INTRODUCTION

Carboxylesterases (CEs, EC 3.1.1.1) are nonspecific hydrolytic enzymes found in organisms ranging from bacteria to humans. CEs are responsible for the metabolism of xenobiotics and endobiotics that contain ester bonds (Cashman et al., 1996). There are two CE isoforms found in human liver, CES1 and CES2. CES1 is also expressed in human monocytes/macrophages, but CES2 is not. On the other hand, CES2 is found in human intestine but CES1 is not. The CEs play an important role in detoxifying oxons (bioactive metabolites of organophosphate pesticides) by forming covalent adducts with these compounds, in addition to hydrolyzing a number of ester-containing substrates including narcotic drugs, therapeutic drugs, and environmental toxicants. Some organophosphate pesticides, such as chlorpyrifos, diazinon, and parathion, do not produce their main toxicity in their original form. An animal's liver can replace a phosphorus-sulfur bond with a phosphorus-oxygen bond, turning these chemicals into oxons. Oxons can inhibit an enzyme (acetylcholinesterases) that breaks down acetylcholine, which is an important neurotransmitter, and this is the primary mechanism of their acute toxicity. Because of their abundance in livers, CEs

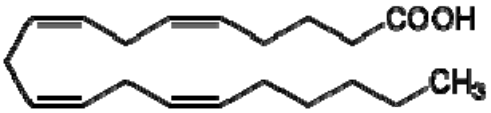
are an important detoxification enzyme for the above organophosphates (Clement and Erhardt, 1990). The most important biological function of CEs may be the hydrolysis and subsequent detoxification of xenobiotics, including pesticides and drugs. However, physiological substrates of CEs are sure to exist but need to be better characterized. For example, CES1 may have an important role in cholesterol mobilization in macrophages, and may catalyze the rate-limiting step of reverse cholesterol transport (Zhao et al., 2007).

The *overall hypothesis* of this thesis is that *endobiotic and xenobiotic inhibitors of carboxylesterases can modulate its function in vivo, which may potentially explain human variation in carboxylesterase hepatic activity and perturb the physiological function of these enzymes.* The first aim of my thesis was to investigate the CE activities in human livers and their interindividual variations. The data obtained showed that 25 individual (healthy) human livers exhibited marked differences in CE activities when using model substrates (*p*-nitrophenyl valerate), pyrethroid insecticides (bioresmethrin), and activity-based proteomic probes (biotin-tagged fluorophosphonate, FP-biotin). However, the difference in CE activities in these individuals was not related to the protein level of CES1, which is by far the most abundant CE isoform in human liver (60-fold greater than CES2; Godin et al., 2007). The levels of CES1 protein in each individual human liver sample are remarkably similar, while CES1 biochemical activities are very different. To account for this, many different hypotheses have been proposed: (1) endogenous inhibitors, such as small molecules, may inhibit

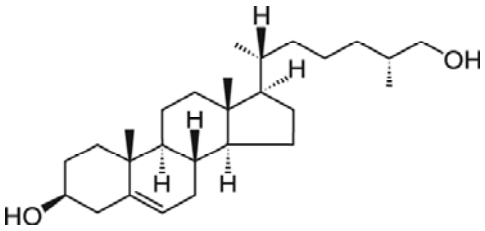
CES1; (2) exogenous inhibitors, such as organophosphate pesticide metabolites, may inhibit CES1; (3) variations in amino acid sequence (polymorphisms) and/or post-translational modifications (PTMs) in CES1 proteins may alter hydrolytic activities. We have been interested to identify potential endogenous inhibitors of CE activity in human liver. Two lipid compounds, arachidonic acid and 27-hydroxycholesterol (see Scheme 1 below), were recently identified by us to be potent endogenous inhibitors of CES1 activity, but not CES2 activity, when using recombinant proteins, cultured cells, and human liver homogenates (Crow et al., 2009). Arachidonic acid is a polyunsaturated fatty acid that is present in both esterified and non-esterified forms in the phospholipid membranes of the body's cells. 27-Hydroxycholesterol, on the other hand, is an oxysterol that acts as a ligand for liver X receptors, which are important regulators of lipid homeostasis. Thus, we hypothesized that the quantities of the endogenous CES1 inhibitors, arachidonic acid and 27-hydroxycholesterol, in individual human livers were inversely correlated with CES1 biochemical activity.

Lipids have structural, energetic, and cell signaling functions. Endocannabinoids and prostaglandins are important classes of lipid mediators. They play essential roles in immune regulation, self-defense, and the maintenance of homeostasis in living systems (Shimizu, 2008). Several lipid mediators contain ester bonds, e.g. the endocannabinoid, 2-arachidonoyl glycerol (2-AG), and the prostaglandin glycerol esters (PG-Gs; PGE_2-G and $PGF_{2\alpha}$-G) (see Scheme 2). 2-AG has been shown to be a selective cyclooxygenase-2 (COX-2) substrate.

Products of COX-2 action on this lipid have been identified as prostaglandin glycerol esters (PG-Gs), which have their own diverse and potent biological activities (Kozak et al., 2001). Endocannabinoids and PG-Gs have been implicated in development and progression of diabetes and atherosclerosis (Sugamura et al., 2009). Identification of the enzymes that degrade lipid mediators and modulate their physiologic actions is important because of the potential to inhibit these enzymes with selective inhibitors for the treatment of human disease. Therefore, the second aim of my thesis investigated whether CEs can degrade the lipid mediators, 2-AG and PG-Gs, in immune cells and to determine if this ability can be inhibited by OP pesticide exposure. The degradation of 2-AG and PG-Gs by recombinant human CEs and cultured cells (human THP1 monocytes / macrophages) that express CES1 was investigated. In addition, inhibition of this metabolism by a selective CES1 inhibitor and by bioactive metabolites of two commonly used OP insecticides was examined.

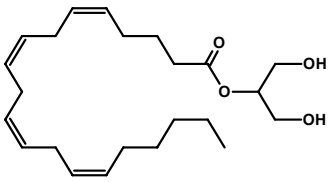


Arachidonic acid (AA)

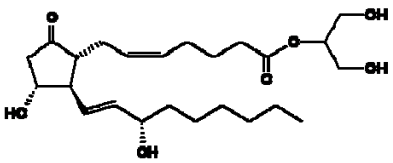


27-Hydroxycholesterol (27-HC)

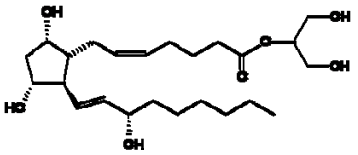
Scheme 1. Endogenous carboxylesterase 1 inhibitors



2-Arachidonoyl glycerol (2-AG)



Prostaglandin E_2-2-glyceryl ester (PGE_2-G)



Prostaglandin $F_{2\alpha}$-2-glyceryl ester ($PGF_{2\alpha}$-G)

Scheme 2. Lipid mediators used as carboxylesterase substrates in this study

CHAPTER II

LITERATURE REVIEW

Carboxylesterases: A promiscuous hydrolytic enzyme

CEs are enzymes that catalyze the hydrolysis of carboxylic esters to their corresponding alcohols and carboxylic acids (Ileperuma et al., 2007), and the conversion of largely lipophilic ester substrates to more water-soluble products, facilitating their elimination (Brzezinski et al., 1997). CEs also efficiently catalyze the hydrolysis of a variety of narcotic and therapeutic compounds, such as cocaine, heroin, CPT-11, temocapril, and benzoic acid derivatives (Taketani et al., 2007). The highest amounts of CE protein are found in the liver (Satoh et al., 2006). CEs are also predominantly involved in hydrolase activity in the small intestine of various mammals. It has been shown using native polyacrylamide gel electrophoresis that CEs are the most abundant hydrolytic enzyme in the liver and small intestine of humans, monkeys, dogs, rabbits and rats (David et al., 1998). Besides animals, carboxylesterases are also widely distributed in plants and microorganisms and catalyze the hydrolytic reactions of various short chain fatty acid esters (Murase et al., 1991).

Most CE enzymes belong to the superfamily of hydrolases with the α/β protein fold, which cleave carboxylesters (RCOOR') by a mechanism that uses a nucleophilic serine present within a Ser-His-Glu catalytic triad (Redinbo and Potter, 2005). In general, carboxylesterases exist as 60 kDa monomers, but a few CEs exist as approximately 180 kDa because they associate to form homotrimers. Liver CEs are N-linked glycoproteins of the high-mannose type. Glycosylation of CEs occurs in the endoplasmic reticulum lumen and stabilizes the active conformation of the protein. The mature CEs are localized to the luminal side of the endoplasmic reticulum. They exist as multiple forms in terms of their isoelectric points and substrate specificities (David et al., 1998). The broad substrate specificities of CEs allow the enzyme to metabolize a wide variety of ester-containing compounds (Brzezinski et al., 1997).

Based on the homology of the amino acid sequence, CEs can be classified into four major groups. The majority of CEs have been identified as members of the CES1 or CES2 family (Hosokawa et al., 2008). In humans and rats, the small intestine only contains enzymes from the CES2 family, while in rabbits and monkeys enzymes from both the CES1 and CES2 families are present (Taketani et al., 2007). CES1 and CES2 family enzymes share ~40–50% amino acid sequence; however, they show very different substrate specificities (Satoh et al., 2002; Imai et al., 2006). The human CES1 catalyzes the hydrolysis of ester substrates that contain relatively "large" acyl groups compared with the alcohol group; for example, temocapril, methylphenidate, and flurbiprofen. In contrast, the hu-

man CES2 isoform typically hydrolyze substrates with a “small” acyl group; for example, CPT-11 and betamethasone valerate. Because the major CEs in human liver and small intestine are, respectively, CES1 and CES2, these organs have remarkably different ester substrate specificities. Temocapril, methylphenidate, and flurbiprofen derivatives can be hydrolyzed in human liver, but not in human small intestine. In contrast, CPT-11 and betamethasone valerate can be efficiently hydrolyzed in human small intestine (Taketani et al., 2007).

The most important biological function of CEs may be the hydrolysis and subsequent detoxification of xenobiotics, including pesticides and drugs. Many pesticides contain organophosphate compounds that bind covalently to the active site of the CE enzymes and inactivate their function (Miller et al., 1999). Because of their abundance in livers, carboxylesterases are an important detoxification route for organophosphates (Clement and Erhardt, 1990).

Arachidonic acid and 27-hydroxycholesterol

Arachidonic acid (AA) is pressed abundently in eukaryotic cells and can be metabolized by cyclooxygenases, lipoxygenases, and cytochrome P450s to produce prostaglandins (PGs), hydroperoxyeicosatetraenoic acid (HPETE), and epoxyeicosatetrenoic acids (EETs), respectively. We have also found that arachidonic acid is a relatively potent inhibitor of CES1 enzyme activity (Crow et al., 2009). The concentration of esterified arachidonate in resting or unactivated human cells (~30 μg arachidonate/10^9 platelets) corresponds to a molar concen-

tration of ~5 mM. Release of 1% of this reserve will give a free arachidonate concentration of 50 μM in the cell. Inflamed tissues often have free arachidonic acid concentrations much higher than 50 μM (Brash, 2001).

27-Hydroxycholesterol (27-HC) is an oxysterol that is the enzymatic product of CYP27A-catalyzed oxidation of cholesterol. Its concentration relative to cholesterol in normal human aorta is about 1.4 mg 27-HC/g cholesterol, which is at least 10-fold greater than what is detected in plasma (Duane and Javitt, 1999). The continual removal of 27-HC, and its metabolites, from vascular tissues into plasma may represent an important component of "reverse cholesterol transport", contributing to overall cholesterol homeostasis (Duane and Javitt, 1999). 27-HC is the most abundant hydroxycholesterol in human circulation (Fu et al., 2001). It circulates in humans at a concentration between 75–730 nM, with ~90% existing in an esterified form (DuSell and McDonnell, 2008).

Endocannabinoids

The endocannabinoid system refers to a group of lipids and their receptors that are involved in a variety of physiological processes including appetite regulation, pain-sensation, lipogenesis, inflammation, mood, and memory. It is named for the endogenous lipids that bind cannabinoid receptors (the same receptors that mediate the psychoactive effects of cannabis). Broadly speaking, the endocannabinoid system refers to: the cannabinoid receptors CB1 and CB2, which are G-protein coupled receptors located in the central nervous

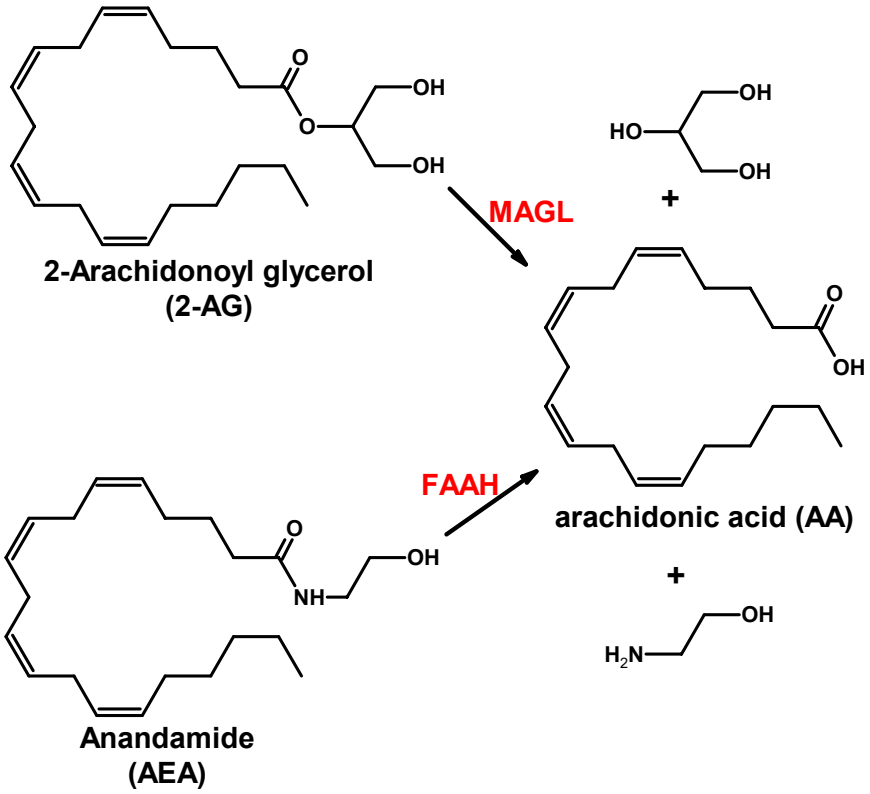
system and periphery; the endogenous arachidonate-based lipids, anandamide (N-arachidonoyl ethanolamine or AEA) and 2-arachidonoylglycerol (2-AG), collectively termed the "endocannabinoids", which are ligands for the cannabinoid receptors; and the enzymes that biosynthesize and degrade AEA and 2-AG. Unlike neurotransmitters, endogenous cannabinoids are not stored in vesicles after synthesis, but are synthesized on demand. Structures of 2-AG and AEA, and their hydrolysis by specific enzymes, are shown below (Scheme 3).

Cyclooxygenase-2 (COX-2) action on arachidonic acid (AA) and the endocannabinoid 2-arachidonylglycerol (2-AG) generates prostaglandins and prostaglandin glyceryl esters (PG-G), respectively. The diversity of PG-Gs that can be formed enzymatically following COX-2 oxygenation of endocannabinoids was previously examined in cellular and subcellular systems (Kozak et al. 2002). Some metabolic reactions that describe their formation and hydrolysis are shown below (Scheme 4).

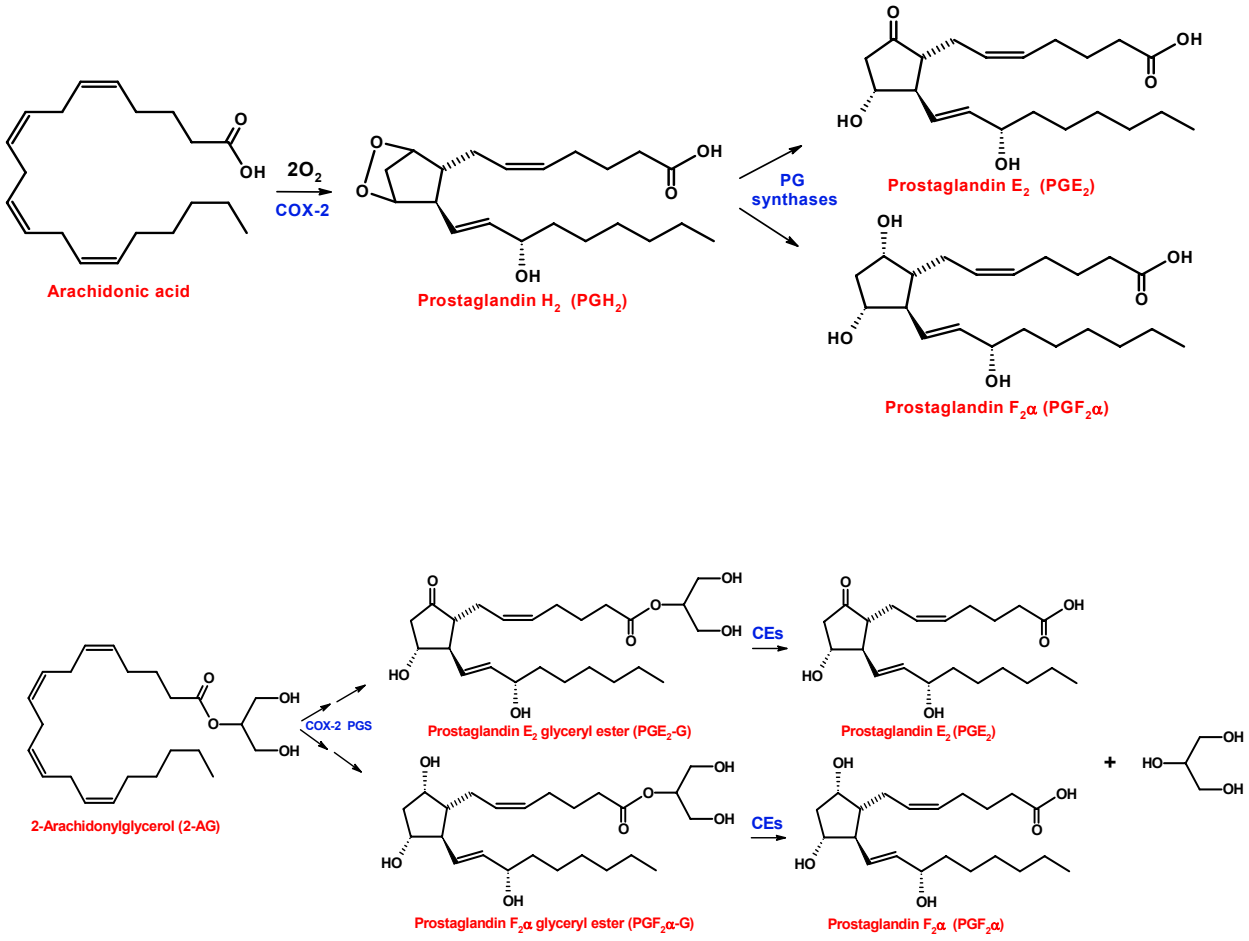
Monoacylglycerol lipase (MAGL)

Monoacylglycerol lipase is a 33-kDa member of the serine hydrolase superfamily. MAGL is a key enzyme in the hydrolysis of the endocannabinoid 2-arachidonoylglycerol in the central nervous system (Dinh et al., 2002). It converts 2-AG to the free arachidonic acid and glycerol. The contribution of MAGL to total brain 2-AG hydrolysis activity has been estimated to be ~85% (Blankman et al., 2007). The compound *N*-arachidonoyl maleimide (NAM) can inhibit MAGL

(Burston et al., 2008). JZL184, which can elevate brain 2-AG levels in vivo, is reported to be an even more selective and potent inhibitor of MAGL than NAM (Long et al., 2009). JZL184 has >300-fold selectivity for MAGL over other brain serine hydrolases.



Scheme 3. Structures of 2-AG and AEA, and their hydrolytic metabolism by monoacylglycerol lipase (MAGL), or fatty acid amide hydrolase (FAAH).



Scheme 4. Metabolic reactions that describe the conversion of arachidonic acid to PGE_2 and $PGF_{2\alpha}$ (top scheme) and the conversion of 2-AG to PG-Gs (bottom scheme) by cyclooxygenase-2.

CHAPTER III

MATERIALS AND METHODS

Reagents, chemicals and materials

Human THP-1 monocytes/macrophages and RPMI-1640 medium were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). Trypan Blue solution (0.4%), acetonitrile, phorbol 12-myristate 13-acetate (PMA), avidin-HRP and all components of the buffers were purchased from Sigma (St. Louis, MO). Paraoxon (PO) and chlorpyrifos oxon (CPO) were synthesized and provided as gifts by Dr. Howard Chambers (Mississippi State University). The CES1 specific inhibitor, S-3030, was a gift from Dr. Phil Potter (St. Jude Children's Hospital, Memphis, TN). Bioresmethrin was obtained from Chem Service (West Chester, PA). N-arachidonoyl maleimide (NAM), arachidonic acid, 27-hydroxycholesterol, prostaglandin E_2-1-glyceryl ester, prostaglandin $F_{2\alpha}$-1-glyceryl ester, 8-*iso*-prostaglandin $F_{2\alpha}$-d_4, 2-arachidonoylglycerol, arachidonic acid-d_8, fatty acid amide hydrolase (FAAH), and monoacylglycerol lipase (MAGL) were purchased from Cayman Chemical (Ann Arbor, MI). Brij 78 was purchased from Acros Organics (New Jersey). Protein A-agarose be

ads were purchased from Invitrogen (Carlsbad, CA). Rabbit pre-immune IgG was purchased from Biomeda Corp (Foster City, CA). FP-biotin was from Toronto Research Chemicals, North York, Ontario, Canada. The enhanced chemiluminescent substrate was from Pierce. Recombinant human CES1 and CES2 proteins were prepared by the method described by Morton and Potter (2000) in Dr. Potter's laboratory.

Cell culture conditions

THP-1 monocytes/macrophages were grown in suspension in RPMI-1640 medium supplemented with 10% FBS, 0.05 mM β-mercaptoethanol, and 50 µg gentamicin/ml (growth medium) in a humidified incubator at 37° C, 95 % air and 5 % CO_2. The cells were cultured at a density between 0.2×10^6 and 1×10^6 cells/ml, as recommended by ATCC.

Preparation of human liver S9 fractions

Twenty-five healthy human livers were obtained from the University of Minnesota *Liver and Tissue Procurement Service*. These livers, from both males and females, were procured from resected portions of livers intended for liver transplants. Liver S9 fractions were prepared as described previously (Ross and Pegram, 2004). S9 includes both the cytosolic and microsomal fractions of tissue homogenates.

Western blot analysis of CES1 in human livers (S9 fractions)

Preliminary experiments determined that 0.2 μg S9 protein was an amount that would produce bands that did not saturate the film. Prior to electrophoresis of human liver S9 fractions, each sample was spiked with an internal protein standard (His-tagged β-actin, 1 μg). For quantitative analysis of CES1 and His-tagged β-actin, PVDF membranes were probed with rabbit anti-CES1 (1:4000) and mouse anti-His (1:5000) antibodies for 1h at room temperature. After washing, membranes were then incubated with goat anti-rabbit HRP and goat anti-mouse HRP secondary antibodies (1:20,000). Chemiluminescent signal were captured on X-OMAT film. Quantitative analysis was done using 1D-Multi software from Alpha Innotech (San Leandro, CA) or Image J (NIH).

Interindividual variation in human hepatic CES1 activity: Bioresmethrin hydrolysis reactions

We used the CES1-selective substrate bioresmethrin (a pyrethroid insecticide), to assay the biochemical activity of the human liver S9 samples. Briefly, bioresmethrin substrate (50 μM) was pre-incubated in 50 mM Tris-HCl buffer (pH 7.4) for 5 min at 37 °C. After pre-incubation, human liver "S9" samples were added to start reactions (total reaction volume, 250 μL) so that the final protein concentration in the reactions was 0.5 mg/mL. After 15 min incubation (37 °C), an equal volume of ice-cold acetonitrile containing the internal standard 3-(4-methoxy)-phenoxybenzaldehyde (10 μM) was added in each tube to terminate

the reaction. The tubes were centrifuged 5 min at 16,000 × g, 4 °C. Supernatants were analyzed by HPLC-UV to assay bioresmethrin hydrolysis products, as described in Ross et al. (2006).

Activity-based probe analysis of CES1 activity in human livers

Fluorophosphonate (FP)-biotin can be used to detect functional CES1 activity in liver S9 samples. Briefly, human liver S9 fractions were suspended in 50 mM Tris-HCl (pH 7.4) buffer (1 mg protein/ml) and incubated with FP-biotin (0.2 uM final concentration) for 0, 1, 3, 5, 7 min or 30 min at room temp in a total volume of 25 μl. FP-biotin is a serine hydrolase activity-based probe (Liu et al., 1999) and was added from a DMSO stock (final DMSO concentration in reactions, 2% v/v). Activity-based probe reactions were terminated by addition of 10 μl of 6x SDS-PAGE loading buffer and were heated at 95°C (5 min). Samples were cooled and immediately loaded on a 10% SDS-PAGE gel. Following electrophoresis, proteins were transferred to a PVDF membrane. After blocking with 3% (w/v) non-fat milk, the membrane was probed with avidin-HRP (Sigma, 1:3000 v/v) for 30-60 min at room temp. The membrane was then washed 4x (~5-10 min each) with Tween buffer, and the Enhanced chemiluminescent substrate (Pierce) was added for 5 min. The chemiluminescent signals on the membrane were captured on x-ray film and band intensities determined by densitometry using Image J (NIH). Plots of normalized integrated band density of native

CES1 versus reaction time were created. Control reactions consisted of proteomes that were heat denatured before treating with FP-biotin.

Arachidonic acid-mediated inhibition of recombinant human CES1

Inhibition of the carboxylesterase activity of human recombinant CES1 by AA was measured by AA's effect on the hydrolysis of *p*-nitrophenyl valerate. Briefly, triplicate reactions were performed in 50 mM Tris HCl (pH 7.4) buffer containing arachidonic acid concentrations varying from 0 - 100 µM in a total reaction volume of 300 µl. Pure recombinant CES1 protein (0.12 µg) was added in each reaction. After pre-incubation of the protein and AA for 5 min at 37° C, reactions were initiated by the addition of 150 µl of freshly prepared 1 mM *p*-nitrophenyl valerate to each well. The reactions were monitored in a plate reader by measuring absorbance (A_{405}) for 5 min.

Activity-based probe analysis of 27-HC- and 22(R)-HC-mediated inhibition of CES1

To study the inhibition of the carboxylesterase activity of human recombinant CES1 by 27-hydroxycholesterol, we designed an activity-based proteomic probe competitive experiment (Leung et al., 2003). Briefly, the recombinant CES1 protein (0.1 µg) was pre-incubated with vehicle (ethanol) or increasing concentrations of 27-HC (0.001, 0.01, 0.1, and 1.0 µM) for 30 min at room temp in a total volume of 25 µl 50 mM Tris-HCl (pH 7.4) buffer. After 30 min, FP-biotin

was added to each sample from a DMSO stock to yield a final concentration of 0.2 μM and allowed to incubate for 7.5 min at room temp (final DMSO concentration, 2% v/v). Activity-based probe reactions were terminated by addition of 10 μl of 6x SDS-PAGE loading buffer and were heated at 95°C (5 min). Samples were cooled and immediately loaded on a 10% SDS-PAGE gel. Following electrophoresis, proteins were transferred to a PVDF membrane and biotin-labeled CES1 protein detected with avidin-HRP as described above. Plots of integrated band density of labeled recombinant CES1 versus 27-HC concentrations were created and the IC_{50} value for 27-HC was determined.

Quantitation of arachidonic acid in human livers

Non-esterified arachidonic acid levels in the human liver samples were determined in the following manner. Approximately 0.025-0.05 g of human liver was weighed into a glass scintillation vial. One-mL of aqueous 1% (w/v) NaCl was added to the weighed human liver samples followed by 2 mL of $CHCl_3$ and 1 mL of MeOH. The livers were homogenized using a Branson sonicator probe on ice in a biosafety hood. After homogenization, 10 μl of 2.6 mM AA-d8 (deuterated arachidonic acid internal standard) was added to the homogenate. The homogenates were centrifuged (3,500 rpm, 10 min) and the organic layer transferred to a clean test tube (16x100 mm). The homogenates were re-extracted with 2:1:2 (v/v/v) $CHCl_3$: MeOH: 1% NaCl/1% formic acid solution. The mixture was centrifuged again and the organic layers were combined and evaporated to dryness

under nitrogen gas. The residues were suspended in 1:1 (v/v) methanol/isopropanol (200 μL), filtered (0.22 μm), and the clear filtrate transferred to LC vials containing a volume reducing insert for targeted LC-MS analysis of arachidonic acid. The endocannabinoids, 2-AG and AEA, could also be analyzed in the same LC-MS run.

Western blot analysis of CES1, FAAH, and MAGL in THP-1 cell lysate

THP-1 monocyte lysate protein was subjected to SDS-PAGE. Following electrophoretic transfer, PVDF membranes were probed with rabbit anti-CES1 (1:4000), rabbit anti-FAAH (1:250), or rabbit anti-MAGL (1:133) for 1h at room temperature. The membranes were then washed followed by incubation with goat anti-rabbit secondary antibody conjugated to HRP (1:20,000). The chemiluminescent signal was recorded using X-OMAT photographic film (Eastman Kodak Co., Rochester, NY).

2-AG and PG-Gs hydrolysis by recombinant CES1 and CES2 protein

2-AG and PG-Gs contain ester bonds, so they might be hydrolyzed by carboxylesterase. Briefly, duplicate or triplicate reactions were performed in 0.01% fatty -acid free bovine serum albumin (BSA) in 50 mM Tris HCl (pH 7.4) buffer containing substrate concentrations varying from 0 - 250 μM (for PG-Gs) and 0 – 400 μM (for 2-AG) in a total reaction volume of 50 μl. After pre-incubation of buffer and substrate for 5 min at 37° C, reactions were initiated by the addition

of 0.2 µg of pure CES1 or CES2 enzyme. The reactions were quenched after a 10 min incubation with an equal volume of acetonitrile containing 2.79 µM of internal standard 8-*iso* prostaglandin $F_{2\alpha}$ -d_4 (for PG-Gs) or 10 µM of internal standard arachidonic acid -d_8 (for 2-AG). Reactions were subsequently placed on ice for ~15 min. Samples were centrifuged at 16,000 x *g* (4° C) for 5 min to remove precipitated protein prior to transferring supernatants into HPLC vials. Supernatants were analyzed for the hydrolysis products prostaglandin E_2, prostaglandin $F_{2\alpha}$, or arachidonic acid by LC-MS methods described previously (Streit et al., 2008).

Kinetic analysis of CE-mediated hydrolysis of 2-AG and PG-Gs

The enzymatic activity versus substrate concentration data was fit by non-linear regression analysis to the Michaelis-Menten equation using Sigma Plot v. 8.02. The kinetic parameters Vmax (maximum velocity), kcat (enzyme turnover number), Km (Michaelis constant), and kcat/Km (catalytic efficiency) were obtained.

Inhibition of CES1 and MAGL in THP-1 monocyte lysates using small-molecule inhibitors

To determine the ability of three small-molecule inhibitors, S-3030, NAM, and JZL184, to inhibit hydrolysis activity in THP-1 monocyte lysates, we used PGE_2-G (for S-3030, NAM, and JZL184), $PGF_{2\alpha}$-G (for JZL184), and 2-AG (for

JZL184) as substrates. For S-3030 and NAM, triplicate reactions contained 60 µg of THP-1 monocyte lysate and S-3030 or NAM (from 0 - 100 µM) in a total reaction volume of 50 µl of 50 mM Tris-HCl (pH 7.4) buffer. After pre-incubation of THP-1 lysate and inhibitors for 30 min at 37° C, reactions were initiated by the addition of 25 µM of PGE_2-G. The reactions were quenched after 30 min with an equal volume of acetonitrile containing 2.79 µM of internal standard 8-*iso* prostaglandin $F_{2\alpha}$-d_4. The reactions were subsequently placed on ice. For JZL184, triplicate reactions contained 27 µg of THP-1 monocyte lysate and S-3030 or NAM (from 0 – 0.5 µM) in a total reaction volume of 50 µl of 50 mM Tris-HCl (pH 7.4) buffer. After pre-incubation of THP-1 lysate and inhibitors for 30 min at 37° C, reactions were initiated by the addition of 25 µM of PG-Gs or 10 µM of 2-AG. The reactions were quenched after a 30 min incubation with an equal volume of acetonitrile containing 2.79 µM of internal standard 8-*iso* prostaglandin $F_{2\alpha}$-d_4 or 10 µM of internal standard arachidonic acid -d_8. The reactions were subsequently placed on ice. Quenched reactions were centrifuged at 16,000 x *g* (4° C) for 5 min to remove precipitated protein prior to transferring supernatants into LC vials. The supernatants were analyzed by LC-MS for the hydrolysis products: prostaglandin E_2, prostaglandin $F_{2\alpha}$, or arachidonic acid (Streit et al., 2008).

Immunoprecipitation of CES1 protein from THP-1 monocyte lysates

THP-1 monocyte lysates were incubated overnight with preimmune IgG (used as control) or anti-CES1 IgG in the presence of protein A-agarose beads to

isolate CES1 protein and deplete it from THP-1 monocyte lysates, as described in detail in Crow et al. (2008).

PG-G hydrolysis by immunodepleted THP-1 monocyte lysates

CES1 protein was immunodepleted from THP-1 monocyte lysates. We used PG-Gs as a substrate to be hydrolyzed by the immunodepleted THP-1 cell lysate. Briefly, triplicate reactions were performed in 50 mM Tris HCl (pH 7.4) buffer containing 25 µM PG-Gs in a total reaction volume of 50 µl. After pre-incubation of buffer and substrate for 5 min at 37° C, reactions were initiated by the addition of 10 µg of control or immunodepleted THP-1 cell lysate. The reactions were quenched after a 30 min incubation with an equal volume of acetonitrile containing 2.79 µM of internal standard 8-*iso* prostaglandin $F_{2\alpha}$-d_4. The reactions were subsequently placed on ice. Samples were centrifuged at 16,000 x *g* (4° C) for 5 min to remove precipitated protein prior to transferring supernatants into HPLC vials. The supernatants were analyzed for the hydrolysis product prostaglandin E_2 or prostaglandin $F_{2\alpha}$ by LC-MS (Streit et al., 2008).

2-AG hydrolysis by recombinant human CES1 and MAGL proteins following treatment with OP oxons

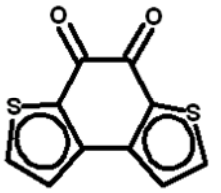
To compare the relative inhibition potency of OP oxons for CES1 and MAGL, the following experiments were designed. Reactions were performed in 50 mM Tris-HCl (pH 7.4) buffer containing OP oxons concentrations varying re-

spectively from 0 – 100,000 nM in a total volume of 50 µl. Pure CES1 or MAGL enzymes (0.5 µg) were added to each reaction. After pre-incubation of enzyme and oxon inhibitor for 15 min at 37° C, reactions were initiated by the addition of 25 µM of 2-AG in each reaction. The reactions were quenched after 15 min with an equal volume of acetonitrile containing 2.5 µM of internal standard arachidonic acid–d_8. The quenched reactions were subsequently placed on ice. Samples were centrifuged at 16,000 x *g* (4° C) for 5 min to remove precipitated protein prior to transferring supernatants into LC vials. The supernatants were analyzed for the hydrolysis product arachidonic acid by LC-MS (Streit et al., 2008).

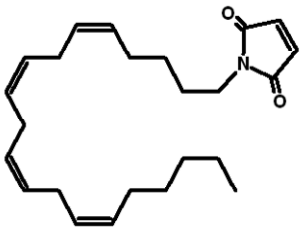
2-AG and PG-Gs hydrolysis by intact THP-1 monocytes following OP treatments

We hypothesized that exposure of human THP-1 cells to OP oxons would inhibit ability of CES1 to degrade prostaglandin glyceryl esters and 2-arachidonoyl glycerol. Test this, cultured THP-1 monocytes (1 x 10^6 cells/ml, 1.0 mL total volume) were placed in six 1.5-mL microfuge tubes in PBS. Paraoxon from an ethanol stock solution was diluted 1:10 in a separate aliquot of fresh culture medium and added to the cells to give the desired final concentration (1.0 µM). The final concentration of ethanol in the medium in all tubes was always 1% (v/v). The monocytes were exposed to the paraoxon for 1 hour. Following paraoxon exposure, 2-arachidonoyl glycerol, prostaglandin E_2-1-glyceryl ester or prostaglandin $F_{2\alpha}$-1-glyceryl ester from the ethanol stock solution was diluted

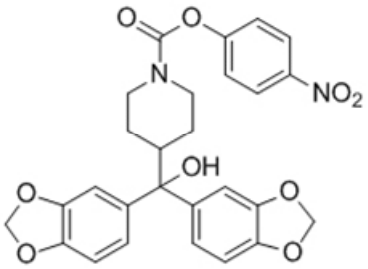
1:10 in a separate aliquot of medium and added to the medium containing the cells to give the desired final concentration (10 μM or 25 μM). The cells were incubated with the lipid mediators for additional 1 hour. After 1 h incubation period was complete, reactions were quenched with an equal volume of acetonitrile (containing 2.79 μM of internal standard 8-*iso*-prostaglandin $F_{2\alpha}$ -d_4 or 10 μM arachidonic acid-d_8) and 3 mL of ethyl acetate containing 0.1% acetic acid. The samples were vortexed for 1 min and then centrifuged at 2,500 x *g* for 5 min. The organic layer (top layer) was collected in glass tubes and evaporated to dryness under N_2. Residues were resuspended in 200 μl of ACN: H_2O (v/v, 1:1) and passed through microfuge filters to clarify the samples. The samples were analyzed for the hydrolysis product prostaglandin E_2, prostaglandin $F_{2\alpha}$, or arachidonic acid by LC-MS (Streit et al., 2008).



S-3030



N-arachidonoyl maleimide (NAM)



JZL 184

Scheme 5. Small-molecule inhibitors: S-3030, NAM, and JZL184

CHAPTER IV

RESULTS AND DISCUSSION

Interindividual variation in human liver CES1 activity

It was previously reported by our laboratory that CES1 protein levels are approximately 60-fold higher than CES2 protein amounts in pooled human liver microsomes (Godin et al., 2007). Furthermore, it was observed that individual human liver microsomes have varying levels of hydrolysis activities when probed with either model ester substrates or ester-containing toxicants (Ross et al., 2006). Therefore, interindividual variation in CES1 protein levels and enzyme activities in 25 individual human (healthy) liver samples was examined in this study. Western blotting was used to measure CES1 protein abundance in S9 liver fractions (contains the cytosolic and microsomal fractions) and enzyme activity was measured by using a pyrethroid insecticide substrate (bioresmethrin), which is hydrolyzed specifically by CES1 (Ross et al., 2006).

Measurement of CES1 protein and biochemical activity in human liver

Increasing amounts of protein from a representative human liver S9 sample were loaded onto an SDS-PAGE gel to determine the appropriate amount for quantitative western blotting (Figure 1). It was determined that 0.2 μg

of liver S9 protein from each individual human liver was the proper amount to load on SDS-PAGE gels because it was within the linear region of the CES1 band intensity vs. S9 protein amount curve (Fig. 1).

Since multiple gels needed to be run on different days, an internal protein standard (His-tagged β-actin, 1 μg) was added to each individual liver sample to control for sample loading and gel-to-gel variation. His-tagged proteins are not produced naturally and can be recognized by specific antibodies that react with the His tag. The relative quantities of CES1 protein were determined by using the ration of the intensity of the CES1 band to the His-tagged β-actin band for each liver sample. Western blots indicated that the 25 individual human liver samples express nearly equal amounts of CES1 protein (Fig 2A). This is based on the fact that the intensity of the protein bands is similar in all liver samples (coefficient of variation, 9%). However, the livers have varying amounts of CES1 biochemical activity (Fig. 2B), based on their ability to hydrolyze a CES1-specific substrate, bioresmethrin (coefficient of variation, 50%). The mere abundance of CES1 protein expression in human liver does not appear to predict its biochemical activity.

This result was confirmed in two ways using the activity based probe, FP-biotin, which reacts covalently with enzymatically active serine hydrolases but not with inactive or denatured proteins (Fig. 3). First, a poor metabolizer (HL1202) and good metabolizer (HL1274) of bioresmethrin were treated with 0.2 μM FP-biotin for increasing time (0-7 min). The intensity of the CES1 band in HL 1202 is faint and similar from 1 to 7 min, while the intensity of the CES1 band in HL1274

is strong and steadily increased from 1 to 7 min (Fig. 4). Second, eight liver samples, including four good metabolizers and four poor metabolizers, were treated with 2 μM FP-biotin and the extent of CES1 labeling determined (Fig. 5). Both heat denatured and native (non-denatured) samples of each individual were treated with the probe. FP-biotin distinguished the functional activity of CES1 in each liver sample. Poor and good metabolizers had faint and strong CES1 bands, respectively. The results shown in Figs. 4 and 5 are consistent with results shown in Fig. 2B and demonstrate that variable CES1 biochemical activities exist in a human population.

Newly identified inhibitors for CES1

While much has been learned about the metabolism of ester-containing xenobiotics by CEs, including how some xenobiotics can inhibit this enzyme, little is known about endogenous inhibitors and substrates that may interact with these proteins and affect their activity.

Inhibition of human CES1 by the endogenous compounds, arachidonic acid and 27-hydroxycholesterol

The levels of CES1 protein in each individual human liver sample are remarkably similar, while their biochemical activities are very different. One possibility to explain these results is the presence of endogenous inhibitors in human liver that might inhibit CES1 activity. Arachidonic acid (AA) and 27-

hydroxycholesterol (27-HC) are two endogenous compounds identified by our laboratory as potent inhibitors of recombinant human CES1 activity (Crow et al., 2009) (Fig. 6 and Fig. 7). K_i values for AA and 27-HC are 1.7 μM and 10 nM, respectively (Crow et al., 2009), indicating that 27-HC is a more potent inhibitor than arachidonic acid. Interestingly, it was found that isomeric oxysterol 22(*R*)-HC does not inhibit CES1 (Fig. 7). Furthermore, both AA and 27-HC inhibit CES1, but not CES2, thus CE isoform selectivity is seen for these inhibitors.

Quantitation of arachidonic acid in human liver

Based on the above findings, we hypothesized that levels of AA and 27-HC in individual human livers may be inversely correlated with CES1 biochemical activity. We measured the amount of free (unesterified) AA in human liver extracts by LC-MS analysis. However, we did not find evidence that the amount of AA present in human livers is inversely correlated with CES1 biochemical activity (Fig. 8, r^2 = 0.0061, p>0.05). Extraction of four S9 fractions (two poor metabolizers and two good metabolizers) instead of whole liver and quantitation of AA by LC-MS also did not indicate that high quantities of AA were present in the poor metabolizers livers and low quantities in the good metabolizers livers (data not shown).

When CES1 specific activities in the 25 human liver samples were divided into tertiles (low tertile, <1000 pmol/min/mg; mid tertile, >1000 pmol/min/mg and <2000 pmol/min/mg; high tertile, >2000 pmol/min/mg) and the lipid composition

(AA and the endocannabinoids, 2-arachidonoyl glycerol and anandamide, which were measured in the same LC-MS analysis) of the three groups were compared, no trends were observed that would suggest that the differential enzymatic activities of the liver samples were related to the liver content of these particular lipids (Fig. 9).

This study has taken a first step in the direction of defining the relationship between the amount of AA and the biochemical activity of CES1 in 25 human liver samples. We focused on AA because it is more abundant than 27-HC in liver and methods that permit analysis of AA using a single-quadrupole mass spectrometer were available to us. The findings, however, go against our hypothesis. Our results demonstrate that AA levels and CES1 biochemical activity in 25 human liver samples are not correlated. A future study might try to quantify hepatic levels of 27-HC because it is a more potent inhibitor of CES1 activity than AA (Crow et al., 2009). In addition, other possibilities to account for interindividual variation in CES1 activity can be postulated, including variations in amino acid sequence (polymorphisms), post-translational modifications (PTMs) in CES1 proteins, protein-protein interactions that can alter the hydrolytic activity of the enzyme or the availability of AA. These should be investigated in the future.

Newly identified substrates for CES1 and CES2

We hypothesized that the natural cannabinoid receptor ligand, 2-arachidonoyl glycerol (2-AG), and its COX-2-derived metabolites, prostaglandin

glyceryl esters (PG-Gs), are substrates for human CEs because each of these compounds contains ester bonds.

Hydrolysis of the endocannabinoid, 2-AG, by human recombinant CES1 and CES2

2-AG can be hydrolyzed by recombinant human CES1 and human CES2 into AA and glycerol (Fig. 10A). LC-MS analysis can measure the velocity of this reaction by measuring the amount of product (AA) formed in a specified amount of time (Fig. 10B). The substrate concentration-velocity data in Fig.10C were fit to the Michaelis-Menten equation [$v = V_{max} \times [S]/(K_m + [S])$] using Sigma Plot and the kinetic parameters were obtained (shown in Table 1). The rate of turnover of 2-AG ($k_{cat} = V_{max}/[E]_0$) by CES1 is higher than CES2; but there is no remarkable difference between the catalytic efficiency (k_{cat}/K_m) parameters for CES1 and CES2 (Table 1).

In contrast to 2-AG, the other endocannabinoid, 2-arachidonoyl ethanolamine (AEA), which contains an amide bond instead of an ester bond, is not hydrolyzed by either CES1 or CES2 (Table 2).

Hydrolysis of PG-Gs by human recombinant CES1 and CES2

$PGF_{2\alpha}$-G and PGE_2-G are also hydrolyzed by human CES1 and CES2 into free prostaglandins, PGE_2 and $PGF_{2\alpha}$ (Fig. 11). Kinetic parameters were determined by fitting data to Michaelis-Menten equation (shown in Table 3). Based on catalytic

efficiencies (k_{cat}/K_m), $PGF_{2\alpha}$-G is a better a substrate for both human CES1 and CES2 than PGE_2-G (Table 2). After inspecting published k_{cat}/K_m values for recombinant rat MAGL (Vila et al., 2007), it appeared that human CES1 and CES2 were better catalysts of PG-G hydrolysis than the rat MAGL. For example, k_{cat}/K_m values for $PGF_{2\alpha}$-G and rat MAGL are 30-89-fold lower than what we observe for CES1 and CES2, while k_{cat}/K_m values for PGE_2-G and rat MAGL are 3-5-fold lower than for CES1 and CES2. We attempted to confirm this finding using recombinant human MAGL enzyme; however, the data revealed a significant species difference between rat and human MAGL activity. When recombinant human MAGL was used to hydrolyze PGE_2-G and $PGF_{2\alpha}$-G, we estimated k_{cat}/K_m values to be 1.6 and 0.31 $min^{-1}\ \mu M^{-1}$, respectively. This data suggests that human MAGL and human CES 1 and 2 have generally similar abilities to hydrolyze PGE_2-G and $PGF_{2\alpha}$-G. But that human MAGL is much more efficient than rat MAGL in hydrolying these esters.

CES1 can hydrolyze 2-AG and PG-Gs because both compounds have ester bonds. PGE_2-G and $PGF_{2\alpha}$-G have very similar structure, but $PGF_{2\alpha}$-G is more efficiently metabolized than PGE_2-G by both CES1 and CES2 recombinant proteins. Compared to CES1, which has a substrate specificity factor of 2 for $PGF_{2\alpha}$-G over PGE_2-G, the substrate specificity factor for CES2 is 13 for $PGF_{2\alpha}$-G over PGE_2-G. One possible explanation for this substrate preference for $PGF_{2\alpha}$-G by human CEs is that the PG-Gs used have two different functional groups that distinguish them: $PGF_{2\alpha}$-G has a β-hydroxyl functional group on the C9 position of the cyclopropane ring, while PGE_2-G has a carbonyl functional group instead at the same position.

The hydroxyl group might cause $PGF_{2\alpha}$-G to have a higher affinity for CES1 and CES2 proteins, perhaps because of a critical hydrogen bond being formed in the active site.

Expression of endocannabinoid hydrolyzing enzymes in THP-1 monocytes

In order to determine if THP-1 monocytes can express fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL) or human carboxylesterase 1 (CES1), we used western blotting to detect them. This was done because FAAH and MAGL are known to hydrolyze endocannabinoids (Vila et al., 2007). From the western blots of THP-1 cell lysates, we can conclude that THP-1 cells express both MAGL and CES1, but not FAAH (Fig. 12). It also should be noted here that CES2 is not expressed in cultured human THP-1 monocytes (Crow et al., 2008). We focus our attention on the human immune cell line, THP-1 monocytes/macrophages, because these cells are a good model for inflammatory mechanisms study. Since several diseases such as atherosclerosis and diabetes have inflammatory components, these cells are useful for the study of disease initiation and progression.

Comparison of the ability of endocannabinoid hydrolyzing enzymes to metabolize 2-AG and PGE_2-G

As mentioned, MAGL and FAAH have been previously reported to hydrolyze 2-AG and PGE_2-G (Vila et al., 2007), but CES1 has not. Therefore, these

three enzymes were directly compared using a single concentration of each substrate (Fig. 13). For 2-AG, the three enzymes hydrolyze the endocannabinoid at similar rates. For PGE_2-G, rank order of hydrolysis rates was as follows: MAGL>CES1>>FAAH. The enzymatic activity of CES1 would appear to be similar or greater than the previously reported MAGL and FAAH.

Inhibition of CES1 and MAGL using small molecules, S-3030 and NAM

Since MAGL and CES1 are both expressed in THP-1 monocytes, small molecule inhibitors of each enzyme were used to determine their relative contribution to the metabolism of 2-AG and PG-Gs.

A cross-inhibition study was performed in which we used 50 μM NAM (MAGL inhibitor) and 50 μM S-3030 (CES1 inhibitor) to pre-treat recombinant human CES1 and MAGL enzymes, respectively. The extent of 2-AG hydrolysis by the enzymes was then determined (Fig. 14). It was found that CES1 is an off-target for NAM (50-60% inhibition of pure enzyme at 50 μM NAM; Fig 14A), while MAGL is not an off-target for S-3030 (Fig. 14B). Thus NAM cannot be called a selective inhibitor of MAGL. We also discovered that JZL 184, another reported selective inhibitor of MAGL, can inhibit CES1 (data not shown).

THP-1 monocyte lysates were then pretreated with increasing concentrations of S-3030 (CES1 inhibitor) followed by addition of synthetic 2-AG (25 μM) (Fig. 15). 2-AG hydrolysis activity in the lysate was reduced by S-3030 in a concentration dependent manner (maximum reduction ~60% at 100 μM S-3030), in-

dicating that CES1 does have a role in metabolizing 2-AG in THP-1 cells. However, complete inhibition was not observed, so another enzyme(s) is likely to also have a role. It is currently not clear what this enzyme is; however, MAGL may be ruled out based on data described below.

We then studied the metabolism of PG-Gs by THP-1 monocytes in the absence or presence of selective inhibitors. First, it was shown that intact cultured THP-1 monocytes could metabolize PGE_2-G and $PGF_{2\alpha}$-G when these compounds were added to the culture medium (Fig. 16). PGE_2-G was hydrolyzed to free prostaglandin to a greater extent than $PGF_{2\alpha}$-G, which is surprising since $PGF_{2\alpha}$-G is hydrolyzed more efficiently by CES1 than PGE_2-G. One possible reason is that $PGF_{2\alpha}$-G may be metabolized by alcohol dehydrogenases more efficiently than PGE_2-G in THP-1 cells, thus reducing the $PGF_{2\alpha}$-G concentration available to be hydrolyzed by CES1.

Second, THP-1 monocyte lysates were pretreated with increasing concentrations of S-3030 or NAM followed by addition of synthetic PGE_2-G (25 μM). The data shows that selective inhibition of CES1 by S-3030 significantly reduces the rate of PGE_2-G hydrolysis catalyzed by THP-1 cell lysate (Fig. 17A); PGE_2-G hydrolysis activity is nearly completely blocked by 100 μM S-3030. Adding the MAGL inhibitor, NAM, to THP-1 cell lysate also reduces PGE_2-G hydrolysis rates, but not to the degree that S-3030 does (Fig. 17B). Because CES1 is an off-target of NAM, it is possible that inhibition seen in Fig. 17B is due to blockage of CES1 activity by NAM.

Hydrolysis of 2-AG and PG-Gs by THP-1 monocyte lysates and intact cells and inhibition of this activity by bioactive metabolites of OP insecticides

Inhibition of recombinant CES1 and MAGL

In this study, we wished to determine the relative sensitivities of recombinant human CES1 and MAGL toward two bioactive insecticide metabolites, paraoxon (PO) and chlorpyrifos oxon (CPO). The recombinant enzymes were pretreated with increasing amounts of PO and CPO for 15 min followed by addition of 2-AG substrate (10 μM). The results show that PO preferentially inhibits CES1 over MAGL, while CPO can inhibit both enzymes (although CES1 appears more sensitive than MAGL) (Fig. 18). Interestingly, even at high concentrations of paraoxon very little inhibition of MAGL results (Fig. 18). According to these results, it is concluded that MAGL is insensitive to PO, but CES1 is not; and both MAGL and CES1 are sensitive to CPO-mediated inhibition.

Hydrolysis of 2-AG and PG-Gs by intact THP-1 monocytes following PO pretreatment

Because PO can inhibit CES1 but not MAGL, this oxon is then an excellent probe to distinguish MAGL and CES1 activities in THP-1 cells. Following pre-treatment of intact THP-1 monocytes with PO (1μM, 30 min), the ability of the cells to hydrolyze exogenous 2AG, PGE_2-G, and $PGF_{2\alpha}$-G (25μM, 1 h) was significantly inhibited (Fig. 19). The remaining hydrolytic activity in the 1 μM PO

group may be attributed to uninhibited (and uncharacterized) enzymes that can hydrolyze these lipid mediators or to incomplete inhibition of native CES1 in the THP-1 monocytes.

Hydrolysis of 2-AG by THP-1 monocytes lysate following PO or CPO pre-treatments

We hypothesized that since MAGL and CES1 are both inhibited by 1000 nM CPO (Fig. 18), but only CES1 by 1000 nM PO, that differential inhibition of 2-AG hydrolysis activity would be observed when THP-1 cell lysate proteins were treated with these oxons. Following pretreatment of THP-1 monocyte lysates with PO or CPO (1μM, 30 min), the ability of the cells to hydrolyze exogenous 2-AG (10μM, 1 h) was significantly inhibited (~50-55%, Fig. 20). However, essentially no difference in inhibition potency was observed for PO and CPO. Thus, the remaining 2-AG hydrolytic activity that is not inhibited by either PO or CPO may be attributed to OP-insensitive enzyme(s). It was previously reported that such an OP-insensitive lipase activity exists in THP-1 monocytes when the fluorogenic substrate 4-methylumbelliferyl oleate was used as the enzyme probe (Crow et al., 2008). The identity of this enzyme remains unknown.

Hydrolysis of PGE_2-G by intact THP-1 monocytes or macrophages following PO pretreatment

Pretreatment of intact monocytes or macrophages with increasing concentrations of PO prior to addition of synthetic PGE_2-G caused concentration-dependent inhibition of PGE_2-G hydrolysis activity in both cell types (Fig. 21). Pronounced inhibition was seen at PO concentrations as low as 100 nM, which indicates that CES1 (but not MAGL) has a role in this metabolism.

Immunodepletion of CES1 protein from THP-1 cell lysate

In this study, THP-1 monocyte lysate was incubated overnight with preimmune IgG (used as control) or anti-CES1 IgG antibodies to immunoprecipitate CES1 protein and deplete the protein from THP-1 monocyte lysate. The western blots of preimmune IgG- and anti-CES1 IgG-treated samples showed that CES1 protein was successfully depleted from the THP-1 cell lysate treated with anti-CES1 antibody (Fig. 22A).

The CES1-immunodepleted lysates were then incubated with 25 μM of each PG-G substrate and hydrolysis product assayed by LC-MS analysis (Fig. 22 B,C). From this data, it appears that CES1 accounts for a major fraction of the PG-G hydrolysis activity in THP1 cell lysates. It also suggests that PGE_2-G is nearly quantitatively metabolized by CES1 in THP-1 cells. The remaining activity in the immunodepleted cell lysates, particularly in the case of $PGF_{2\alpha}$-G, is possibly due to presence of other enzymes that can metabolize PG-Gs.

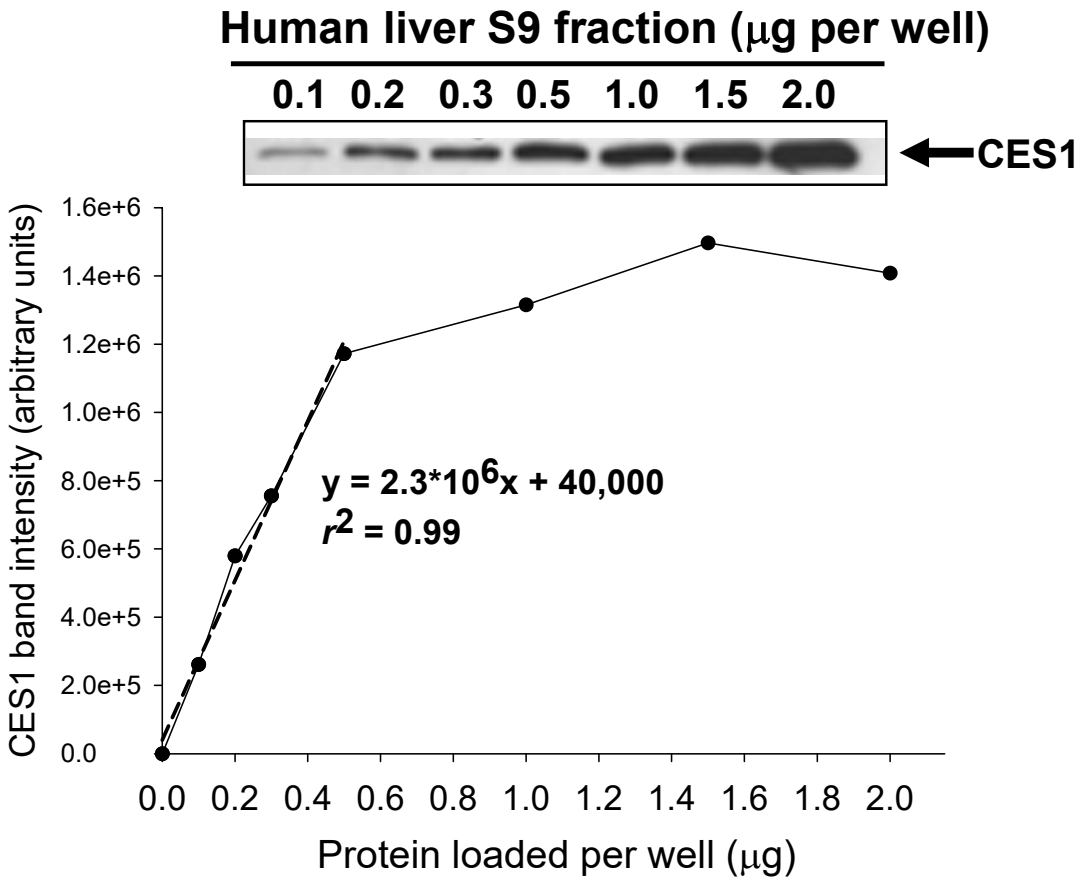


Figure 1. Western blot of CES1 protein in titrated human liver S9 fraction.

Increasing amounts (0.1-2.0 μg) of denatured human liver S9 protein were loaded in each well of an SDS-PAGE gel. The intensity of the immunoreactive bands was measured and a CES1 band intensity vs. protein amount plot was made. The dashed line indicates the linear regression curve (equation and correlation coefficient are shown). According to the curve, 0.2 μg of S9 protein was within the linear region.

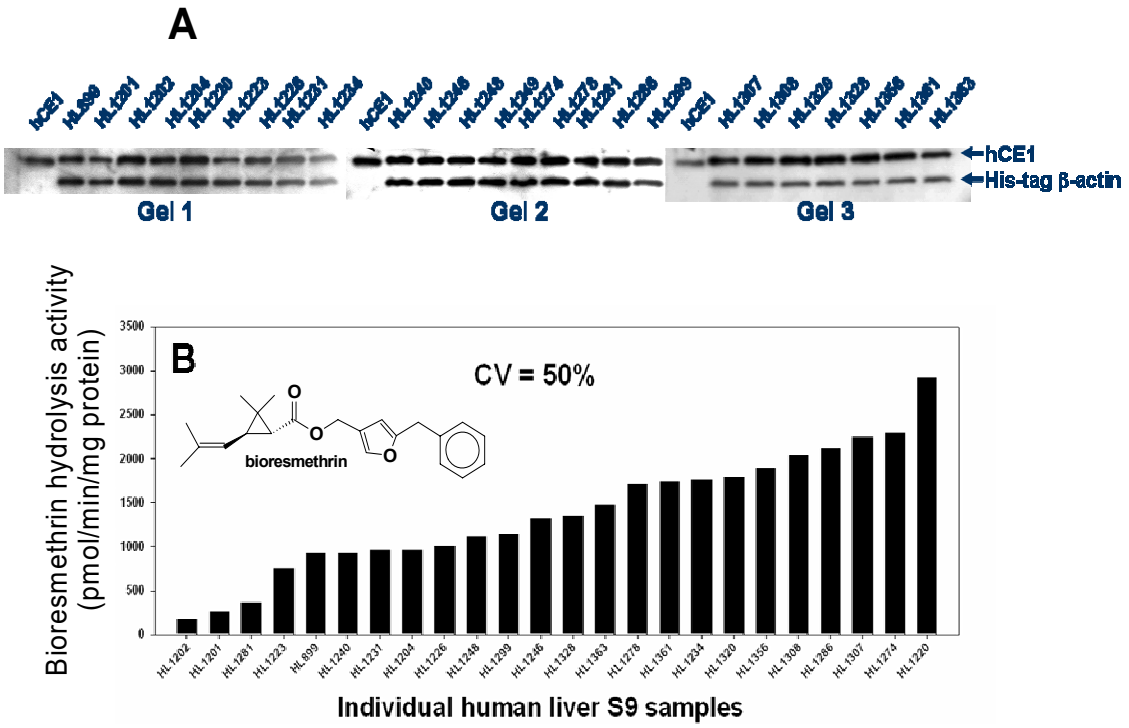


Figure 2. The mere abundance of CES1 protein expression in human liver does not predict its biochemical activity.

(A) Human liver S9 proteins were subjected to SDS-PAGE analysis and immunoblotting using anti-CES1 and anti-His antibodies. A similar quantity of His-tagged β-actin (1 μg) was added to each sample before electrophoresis for use as internal standard and to control inter-gel variations. (B) Human liver S9 samples were used to determine their hydrolytic activity against a CES1-specific substrate, bioresmethrin. This substrate is not hydrolyzed by CES2, only CES1. Bioresmethrin hydrolysis reactions using human S9 fractions were performed in 50 mM Tris-HCl buffer (pH 7.4) after adding 2.5 μL of 5 mM fresh bioresmethrin. Reactions were terminated after 15 min with 250 μL acetonitrile containing an internal standard (10 μM 3-(4-methoxy)-phenoxybenzaldehyde). After centrifugation, the supernatant was analyzed by HPLC/UV. All reactions were performed in triplicate and specific activities are reported as pmol/min/mg protein (CV<15% for each sample). The specific activity of each individual liver sample is plotted in the order from poorest metabolizer to greatest metabolizer of bioresmethrin.

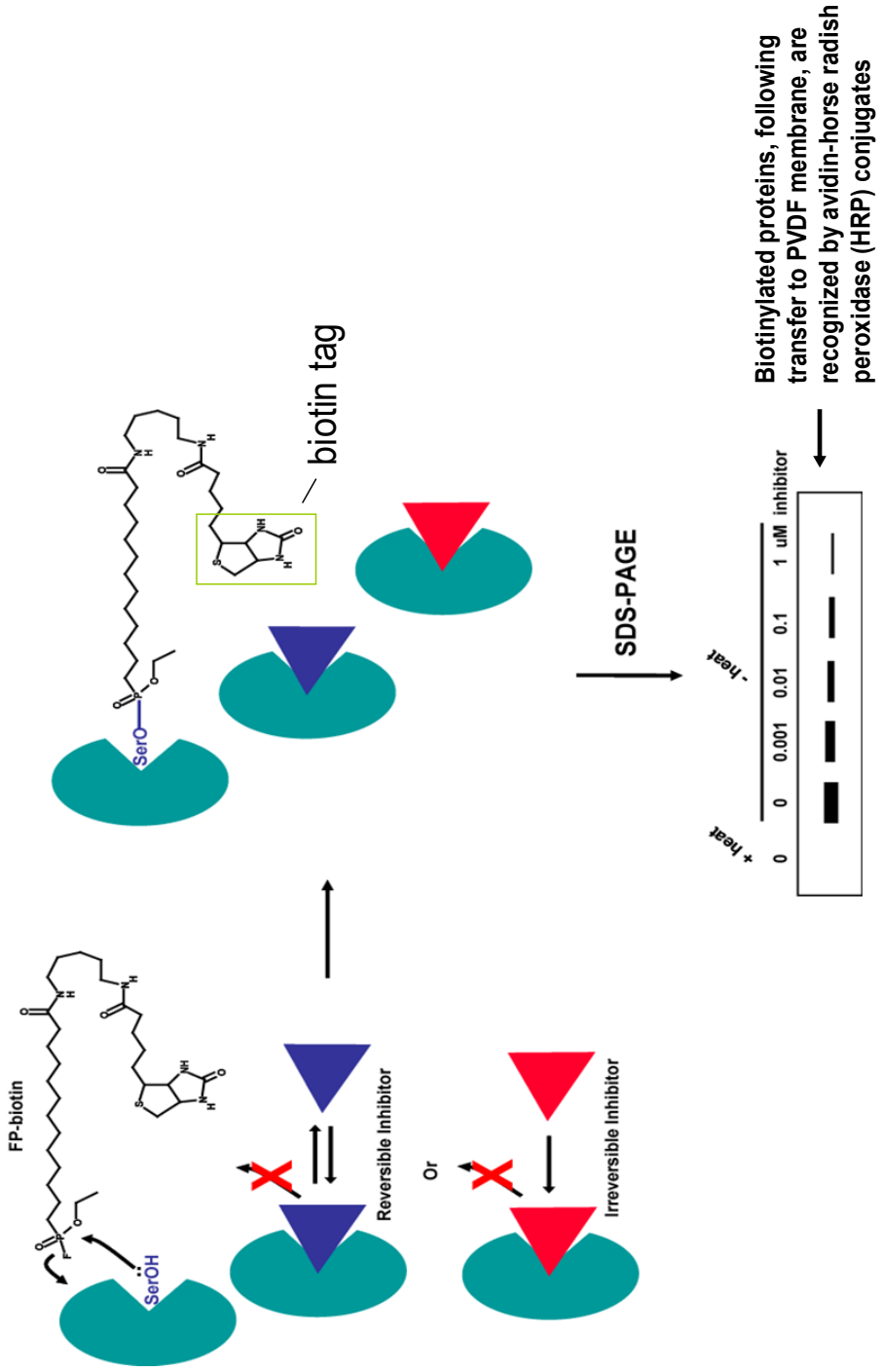


Figure 3. Inhibition of serine hydrolase family of enzymes by reversible or irreversible inhibitors can be evaluated using the activity-based probe, fluorophosphonate (FP)-biotin.

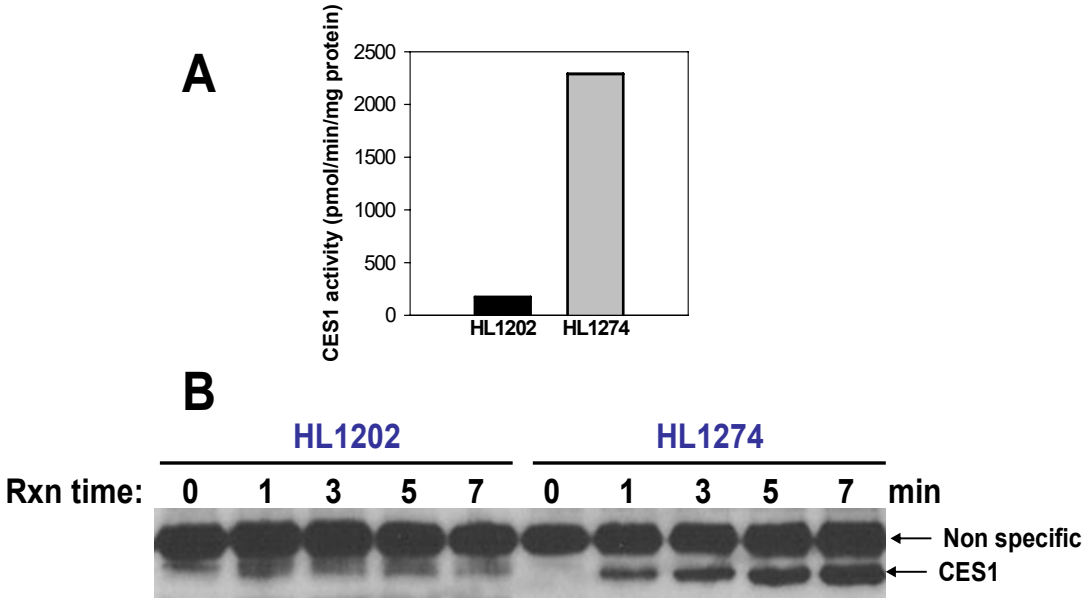


Figure 4. Time-course of reaction between FP-biotin and two human liver samples (HL1202 and HL1274).

(A) HL1202 and HL1274 are poor and good metabolizers of bioresmethrin, respectively (same data as presented in Fig. 2B). (B) Avidin-HRP blotting of biotinylated proteins following a 0–7 min reaction of the two liver samples with 0.2 μM FP-biotin. The CES1 protein is indicated with an arrow. The arrow designated as "Non specific" refers to proteins that are not enzymatically active, but are still labeled by FP-biotin.

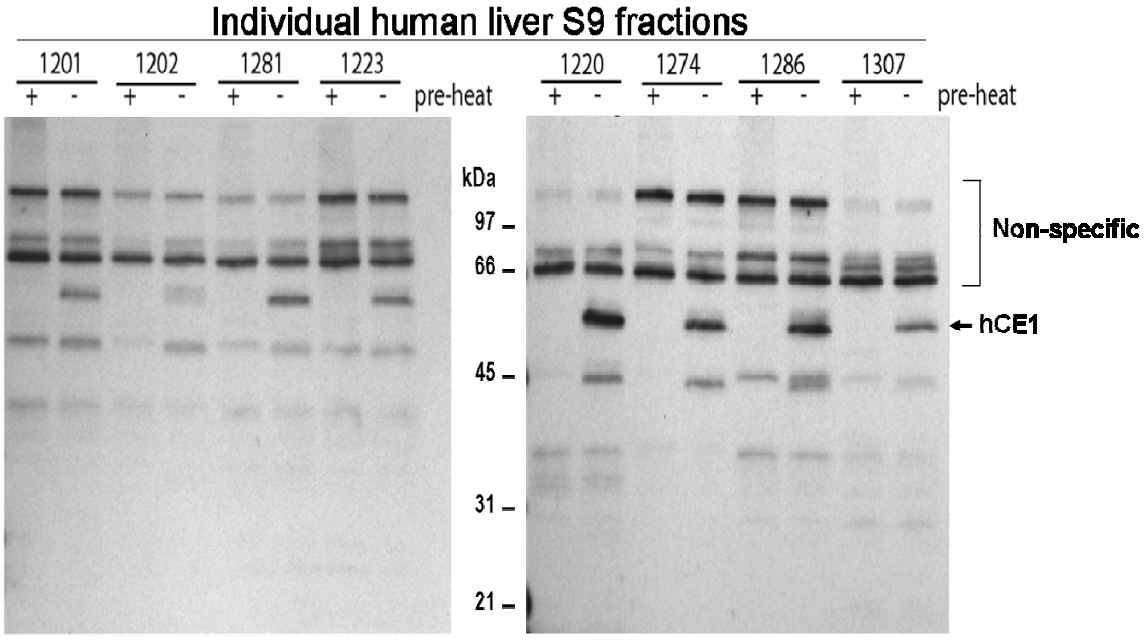


Figure 5. Fluorophosphonate (FP)-biotin can distinquish the functional activity of CES1 in complex proteomes. The intensity of the hCE1 (CES1) band in each human liver S9 sample is related to its intrinsic biochemical activity.

Liver proteins (1 mg/ml) were treated with 2-μM FP-biotin for 1 h at room temperature. The quenched reactions were subjected to SDS-PAGE, transferred to PVDF membranes and probed with avidin conjugated to HRP to detect FP-biotin-labeled proteins. Note the difference between sample 1202 (a poor metabolizer) and 1274 (a good metabolizer). The + pre-heat labeled bands correspond to proteins that react with FP-biotin after heat denaturation of liver proteins, which result from non-specific binding of FP-biotin to protein. “Non specific” refers to proteins that are not enzymatically active, but are still labeled by FP-biotin in the absence or presence of heat denaturation.

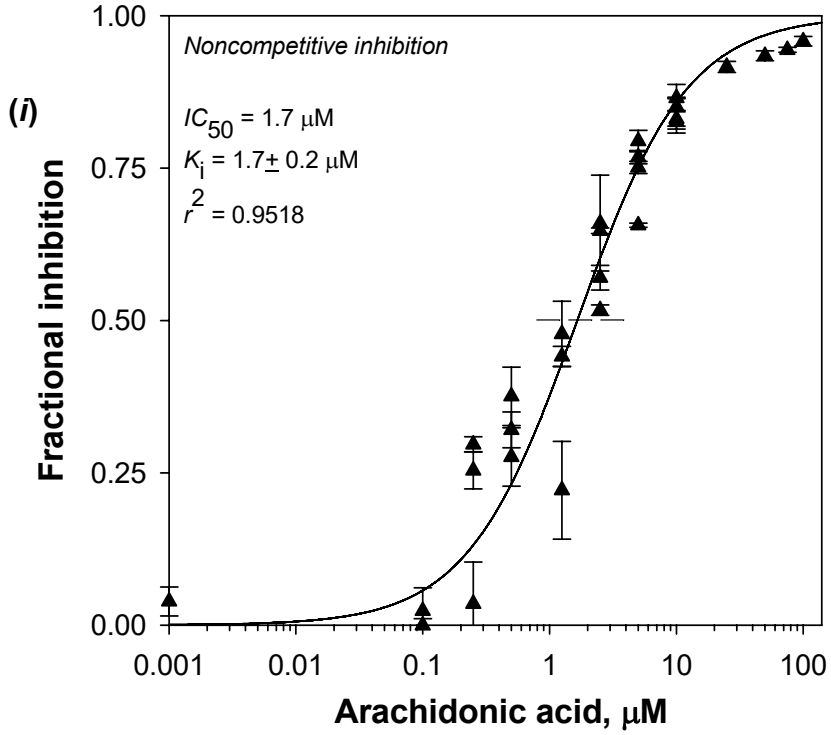


Figure 6. Inhibition of the carboxylesterase activity of human recombinant CES1 by AA. Hydrolysis of *p*-nitrophenyl valerate by CES1 in the presence of arachidonic acid was measured by a spectrophotometric assay.

The data were fit to an equation that describes noncompetitive enzyme inhibition (Webb, 1963): $i = [I]/([I] + K_i)$. The combined data from 3-4 independent experiments are shown (error bars, SD, represent the intra-assay variation for each independent experiment).

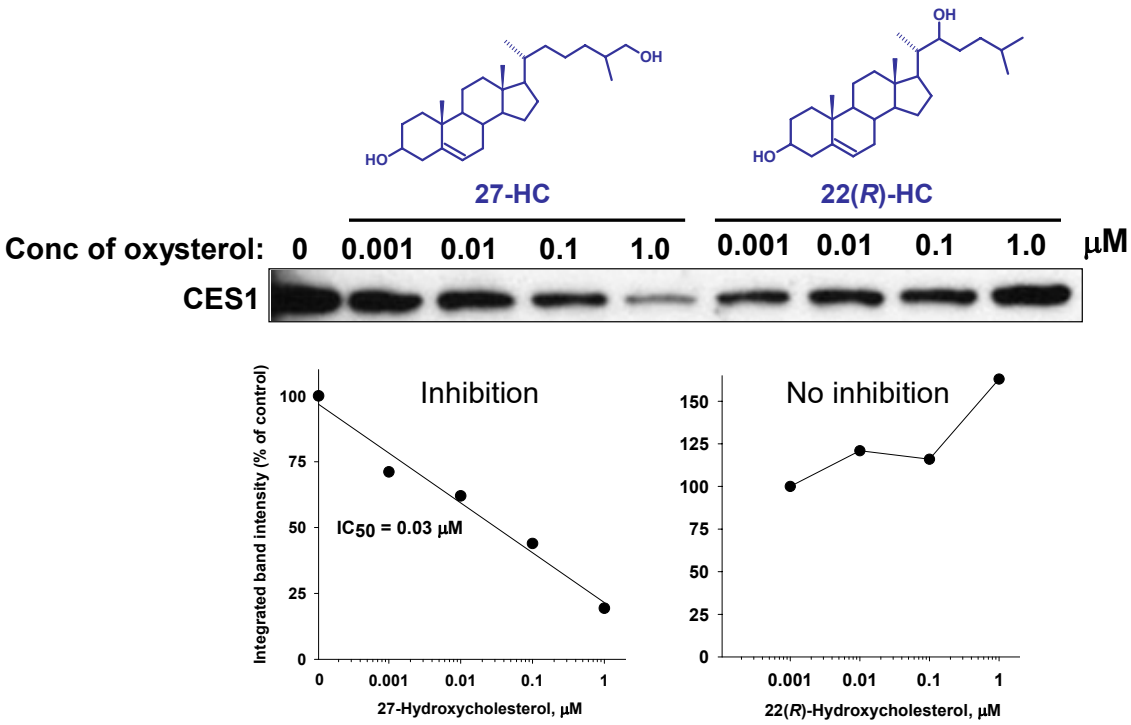


Figure 7. Activity blots of recombinant CES1 following pretreatment with 27-HC or 22(*R*)-HC. 27-HC is a potent inhibitor of CES1 (*Left*), but 22(*R*)-HC is not (*Right*).

Inhibition of the carboxylesterase activity of human recombinant CES1 by 27-hydroxycholesterol, but not 22(*R*)-hydroxycholesterol: Reaction of CES1 with 0.2 µM FP-biotin after pretreatment with increasing concentrations of 27-HC and 22(*R*)-HC.

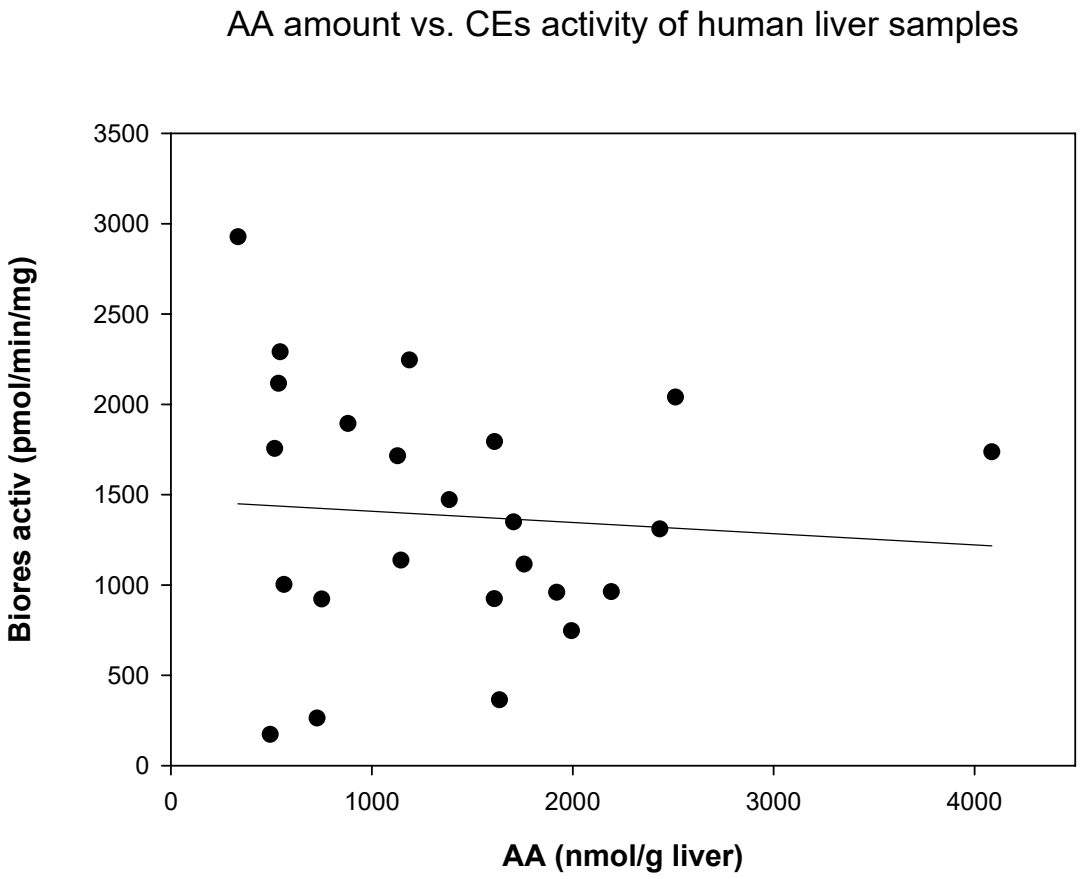


Figure 8. The levels of arachidonic acid (AA) vs. CES1 activity in human liver samples.

AA was extracted from human liver samples and then analyzed by LC-MS and an AA vs. bioresmethrin activity (CES1 activity) curve was made. According to the curve, the amount of arachidonic acid present in human livers were not correlated with CES1 activity (r^2 = 0.0061, p>0.05).

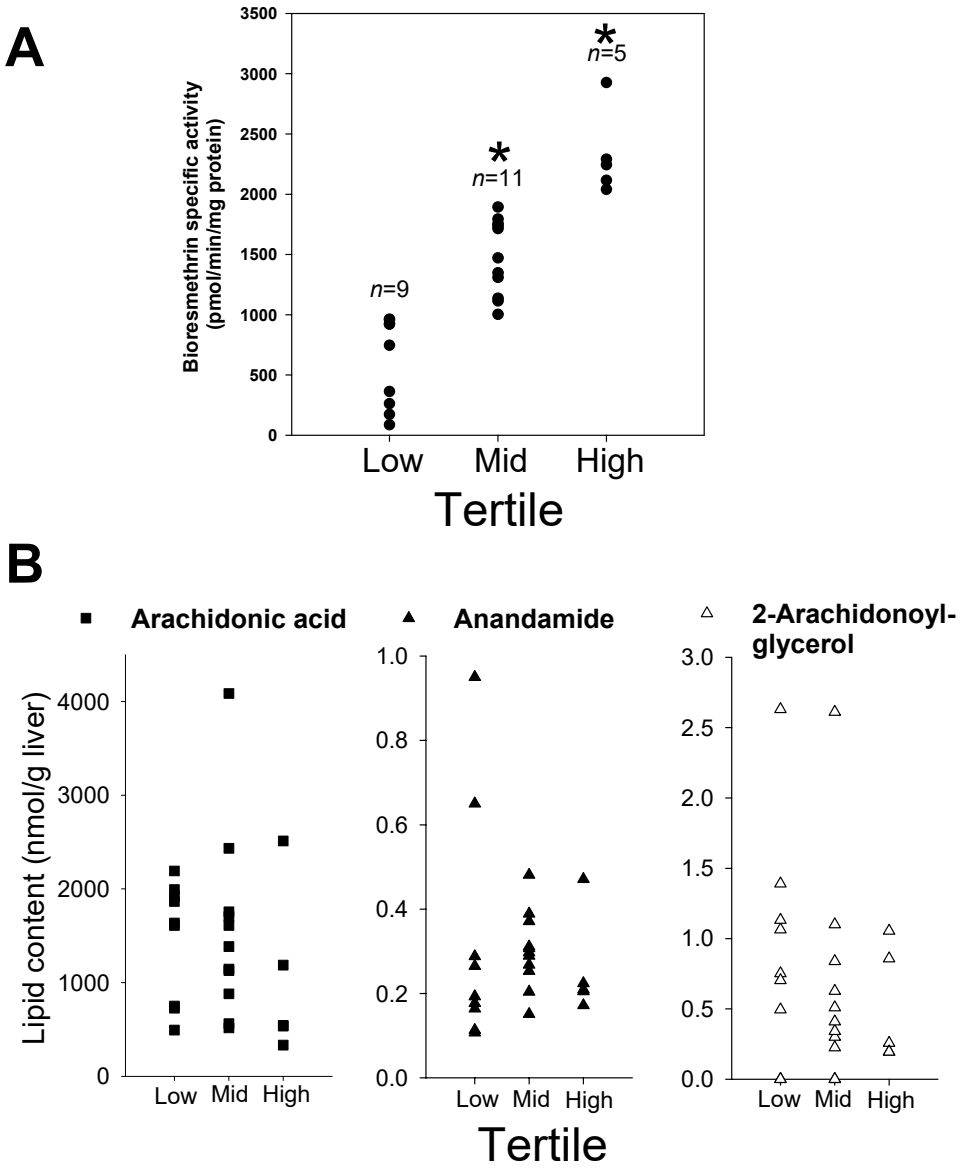


Figure 9. Content of arachidonic acid and endocannabinoids in human livers

(A) The CES1 specific activities (determined using the pyrethroid insecticide, bioresmethrin) of the liver samples were divided into three groups (tertiles). Significant differences in enzymatic activities were noted when the mid and high tertile groups were compared to the low tertile group (* $p<0.05$, one-way ANOVA and Dunnett's test). (B) The quantities of arachidonic acid and endocannabinoids (anandamide and 2-arachidonoyl glycerol) in the liver samples did not parallel the CES1 hydrolytic activity in the three tertile groups.

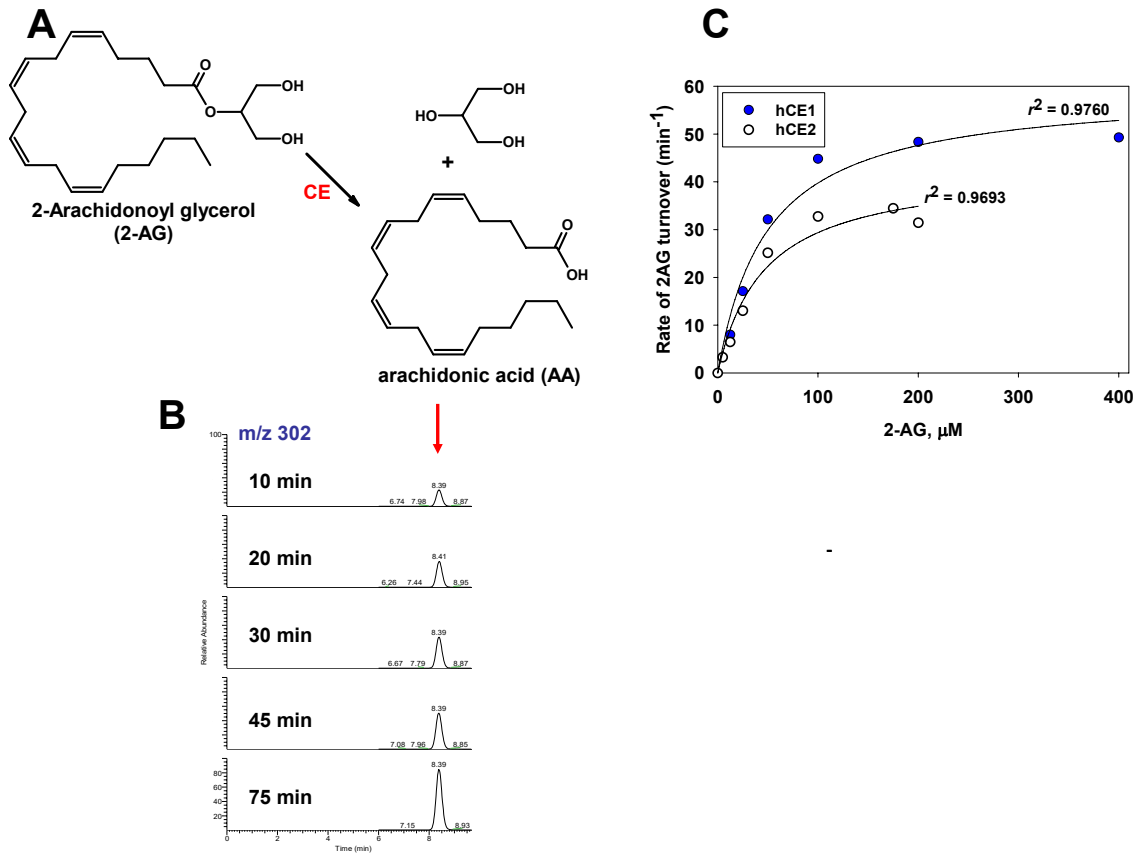


Figure 10. 2-arachidonoyl glycerol hydrolysis by recombinant human CES1 and CES2.

(A) Chemical scheme of the 2-AG hydrolysis reaction catalyzed by human CEs. (B) The amount of arachidonic acid (AA) product formed between 10 and 75 min incubation of 2-AG with CES1 was determined by LC-MS analysis (m/z 302, negative ion mode). (C) Substrate concentration vs. velocity (rate of turn-over of 2-AG) curves for human recombinant CES1 and CES2. Recombinant human CES1 and CES2 (0.2 μg/reaction) were incubated with 2-arachidonoyl glycerol for 20 min at 37°C in 50 mM Tris-HCl (pH 7.4) containing 0.01% w/v fatty-acid free BSA. Kinetic parameters for each enzyme, determined by fitting the data to the Michaelis-Menten equation, are presented in Table 1 below.

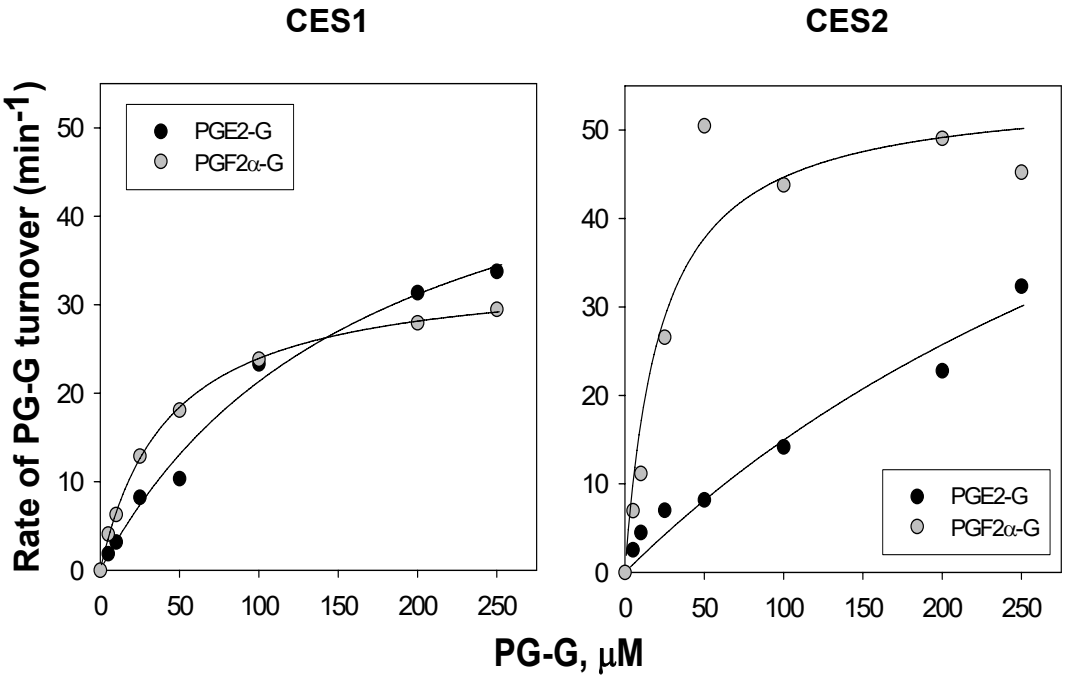


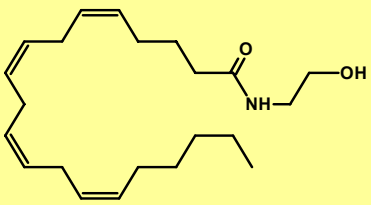
Figure 11. Substrate concentration vs. velocity (rate of turnover of PGE_2-G or $PGF_{2\alpha}$-G) curves for recombinant human CES1 and CES2.

Recombinant human CES1 (*left panel*) and CES2 (*right panel*) (0.2 μg/reaction) were incubated with the prostaglandin glyceryl esters, PGF2α-G or PGE2-G, for 10 min at 37°C in 50 mM Tris-HCl (pH 7.4) containing 0.01% w/v fatty-acid free BSA. Kinetic parameters for each enzyme, determined by fitting the data to the Michaelis-Menten equation, are presented in Table 3 below.

Table 1. Steady-state Michaelis–Menten kinetic parameters for 2-arachidonoyl glycerol hydrolysis by human recombinant CES1 and CES2.

Steady-state Kinetic Parameters			
Carboxylesterase isozyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
CES1	59 ± 4	49 ± 11	1.2
CES2	43 ± 4	46 ± 13	0.9

Table 2. Summary of enzymes that can hydrolyze the endocannabinoids, 2-arachidonoyl glycerol (2AG) and anandamide (AEA).



Anandamide (AEA)

Substrate	MAG Lipase	FAAH	CES1	CES2
2-AG	+	+	+	+
AEA	–	+	–	–

+, activity detected; –, no activity detected

***In vitro* catalytic efficiencies (μM^{-1} min^{-1})**

Substrate	MAG Lipase	FAAH	CES1	CES2
2-AG	2.1	18.6	1.2	0.9
AEA	–	27.6	–	–

Data for MAG Lipase and FAAH are from Vila et al. (2007) *Biochemistry* 46: 9578-9585.

Abbreviations:
MAG, monoacylglycerol
FAAH, fatty-acid amide hydrolase
hCE1, human carboxylesterase 1
hCE2, human carboxylesterase 2

Table 3. Steady-state Michaelis–Menten kinetic kinetic parameters for PG-G hydrolysis by human recombinant CES1 and CES2.

	Human CES1		
Substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
$PGF_{2\alpha}$-G	34.2	42.7	0.80
PGE_2-G	57.9	171.7	0.34
	Human CES2		
Substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
$PGF_{2\alpha}$-G	54.7	22.4	2.4
PGE_2-G	92.6	520	0.18

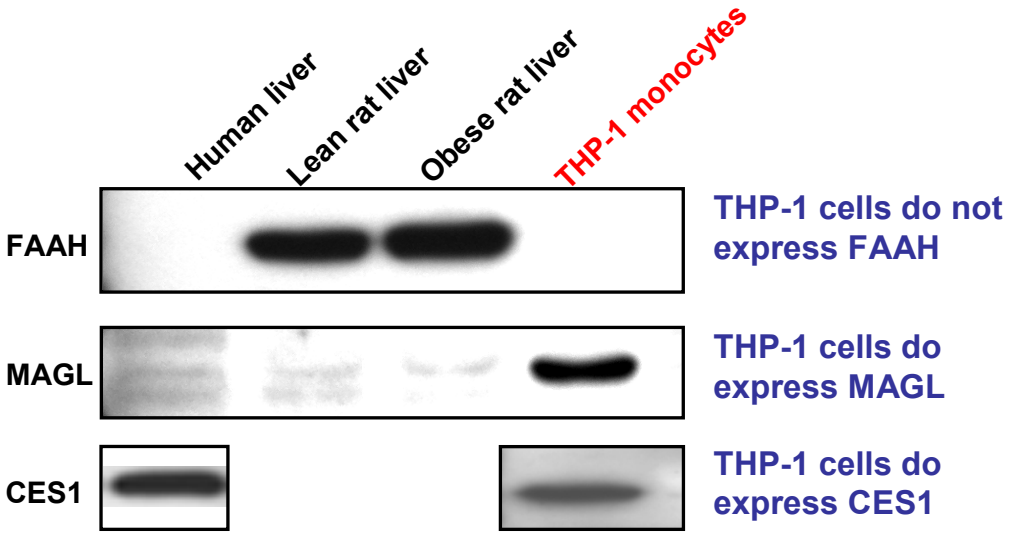


Figure 12. THP-1 monocytes express CES1 and MAGL, but do not express FAAH.

In this western blot analysis, PVDF membranes were probed for CES1, MAGL, and FAAH proteins using rabbit anti-CES1 [1:4000 (v:v)], rabbit anti-MAGL [1:133 (v:v)], or rabbit anti-FAAH [1:250 (v:v)]. Antigen-antibody complexes were detected using goat anti-rabbit secondary antibody conjugated to HRP [1:20,000 (v:v)].

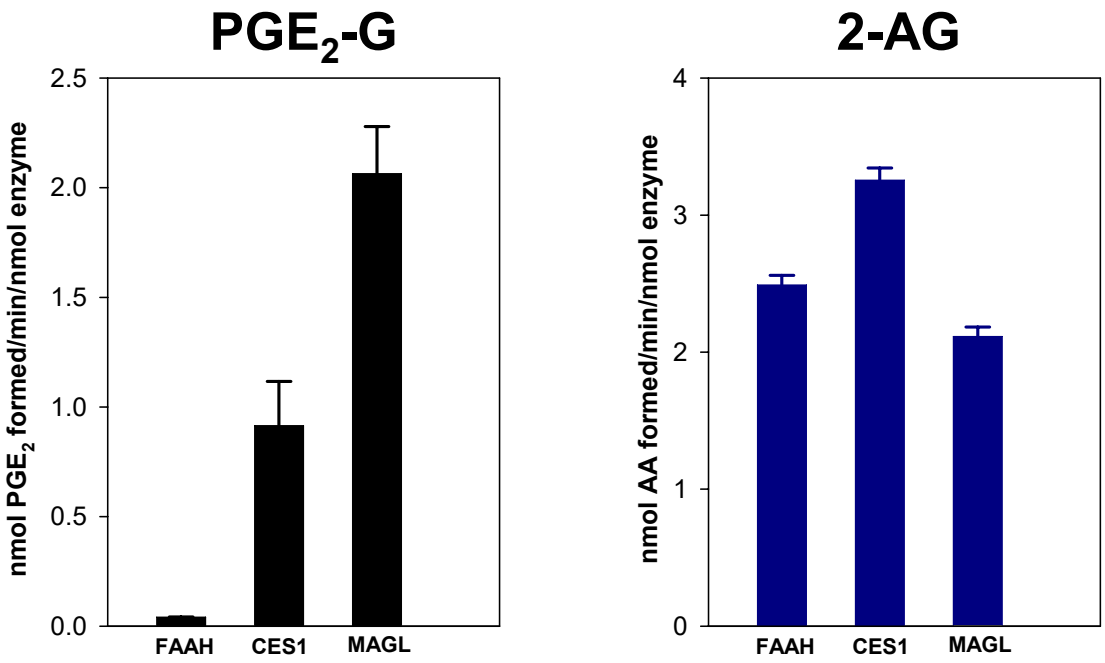


Figure 13. Comparison of FAAH, CES1, and MAGL hydrolytic enzymes and their ability to metabolize PGE_2-G (25 μM) or 2-AG (10 μM).

Recombinant human enzymes (0.5 μg/reaction) were incubated with substrate for 15 min. Products are analyzed by LC-MS and quantified with internal standard (8-iso-PGF2a-d_4 or AA-d_4). Data represent the mean ± SD of triplicate reactions.

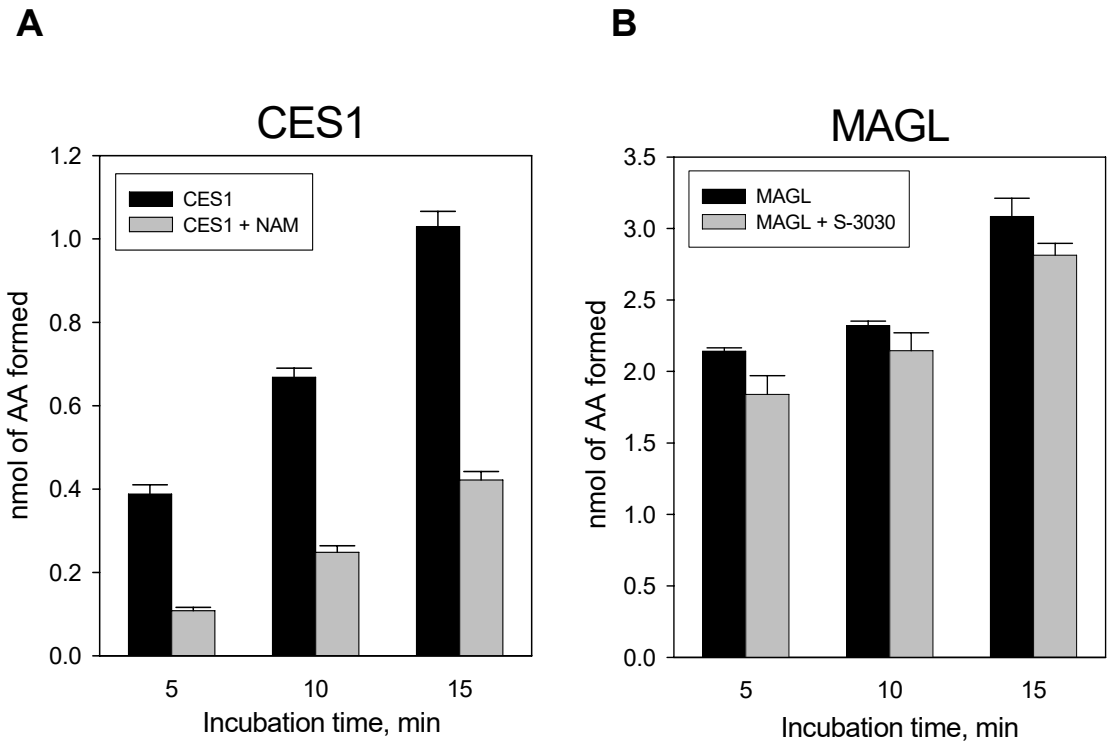


Figure 14. N-arachidonoyl maleimide (NAM, 50 μM) cross-inhibits recombinant human CES1 (A), but S-3030 (50 μM) does not inhibit recombinant human MAGL (B).

The substrate used in this experiment was 2-arachidonoyl glycerol (25 μM final concentration). The amount of arachidonic acid (AA) was determined by LC-MS analysis and quantified with an internal standard (AA-d_4). The extent of product formation was determined 5, 10, and 15 min after the reaction started. Data represent the mean ± SD of duplicate reactions.

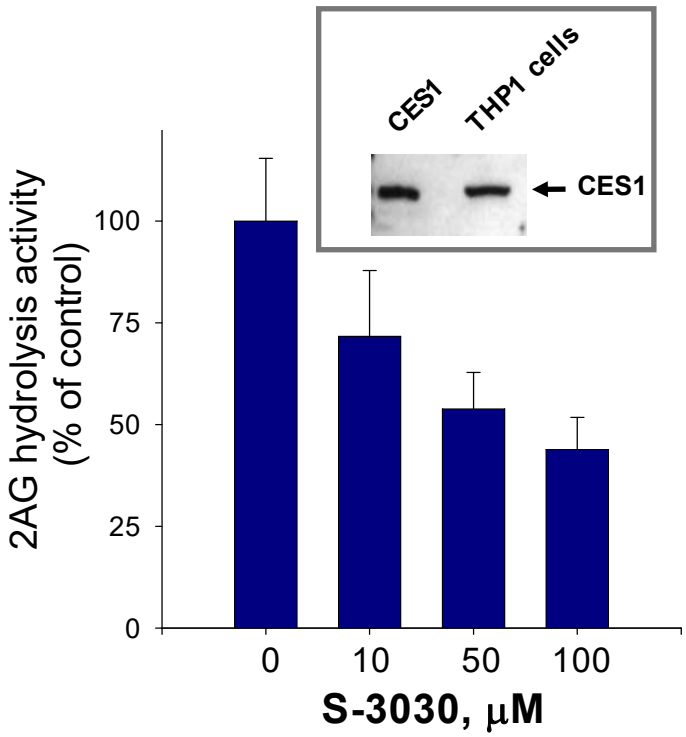


Figure 15. Selective inhibition of CES1 activity in THP1 monocyte lysates reduces 2-AG hydrolysis rate.

Inset, western blot of recombinant CES1 protein (left lane) and THP-1 cell lysate (right lane) probed with anti-CES1 antibody. *Bar graph*, THP-1 cell lysate was pre-incubated with increasing concentrations of the CES1-selective inhibitor S-3030 before adding 2-AG (25 μM). The amount of arachidonic acid was quantified by LC-MS using the AA-d4 internal standard.

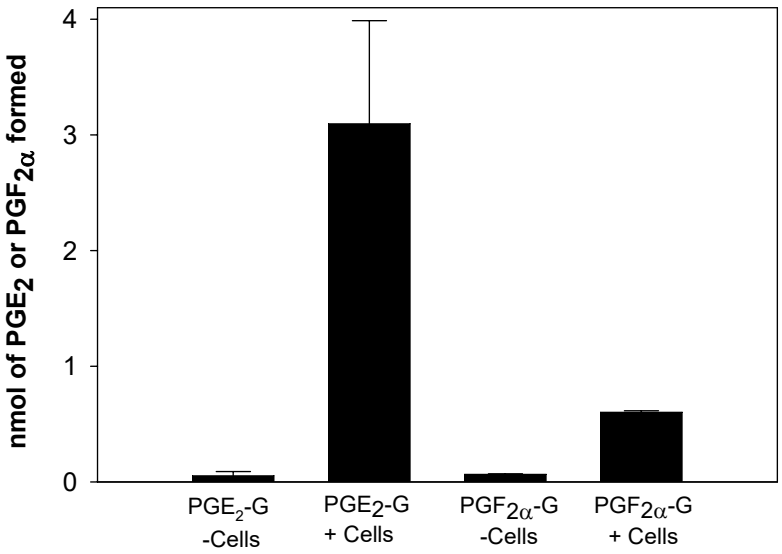


Figure 16. Cultured THP-1 monocytes can metabolize synthetic PGE_2-G and $PGF_{2\alpha}$-G to free prostaglandins.

Data represent the mean ± SD of triplicate reactions. – cells, PG-G was added to FBS-free culture medium only; + cells, PG-G added to FBS-free culture medium + THP-1 monocytes.

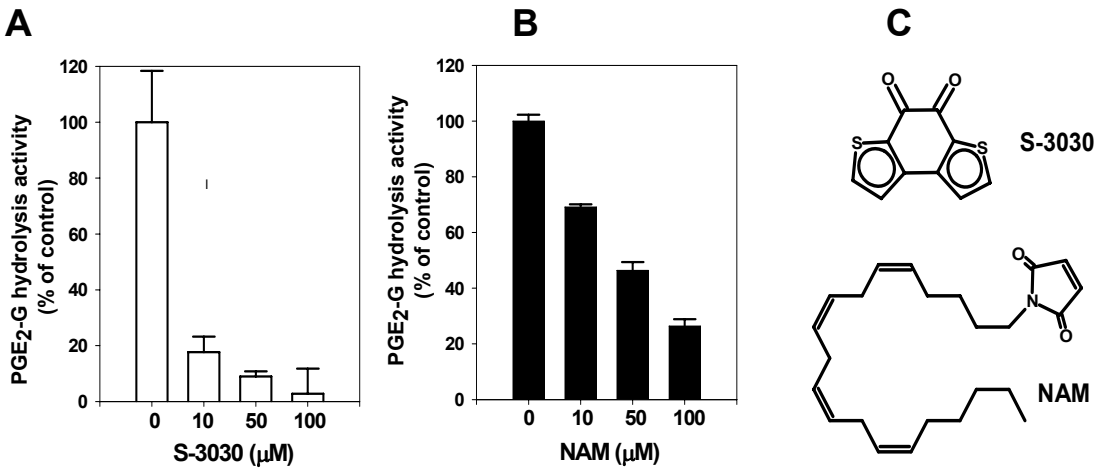


Figure 17. Inhibition of CES1 and MAGL in THP-1 monocyte lysates using the small molecules, S-3030 and NAM

Addition of CES1-selective inhibitor S-3030 to THP-1 monocyte lysates reduces hydrolysis of PGE_2-G (A), but addition of a monoacylglycerol lipase (MAGL) inhibitor, NAM, is less effective (B). Structures of S-3030 and NAM are shown in (C). Data represent the mean ± SD of duplicate or triplicate reactions.

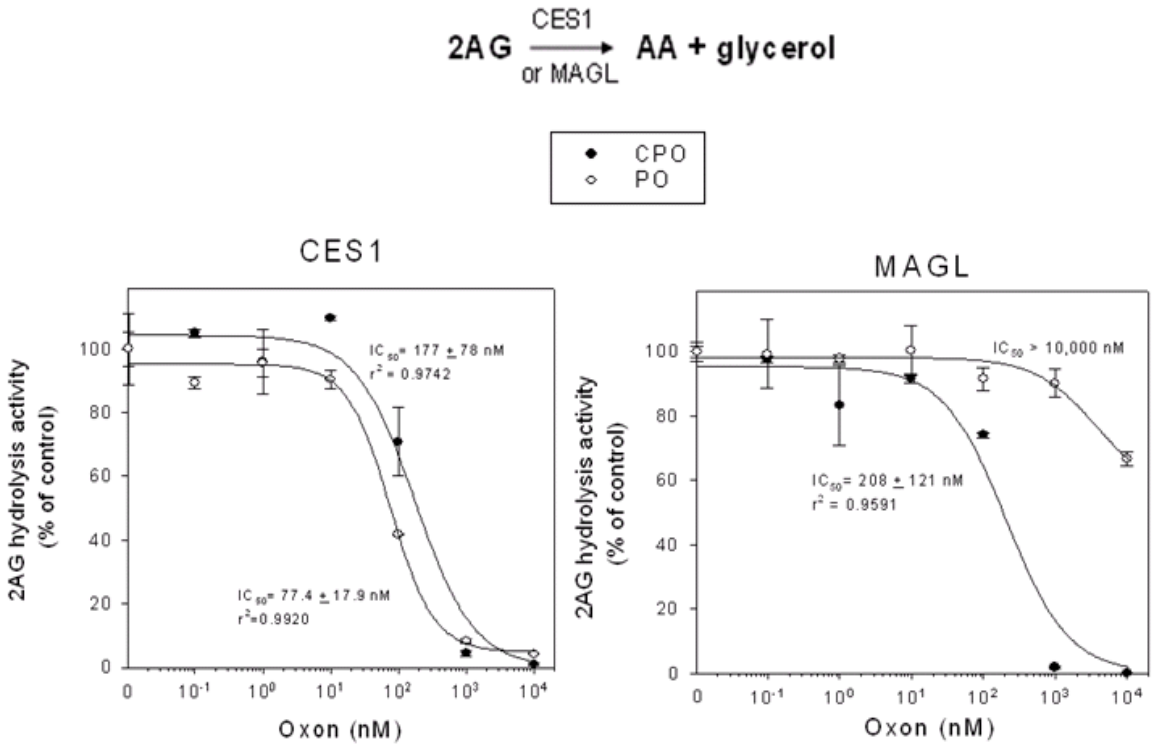


Figure 18. Inhibition of 2-AG hydrolysis catalyzed by recombinant human CES1 and MAGL following pretreatment with paraoxon and chlorpyrifos oxon.

Paraoxon preferentially inhibits CES1 over MAGL, while chlorpyrifos oxon can inhibit both enzymes although CES1 appears to be more sensitive than MAGL. Even at high concentrations of paraoxon, very little inhibition of MAGL results. Data represent the average of duplicate reactions.

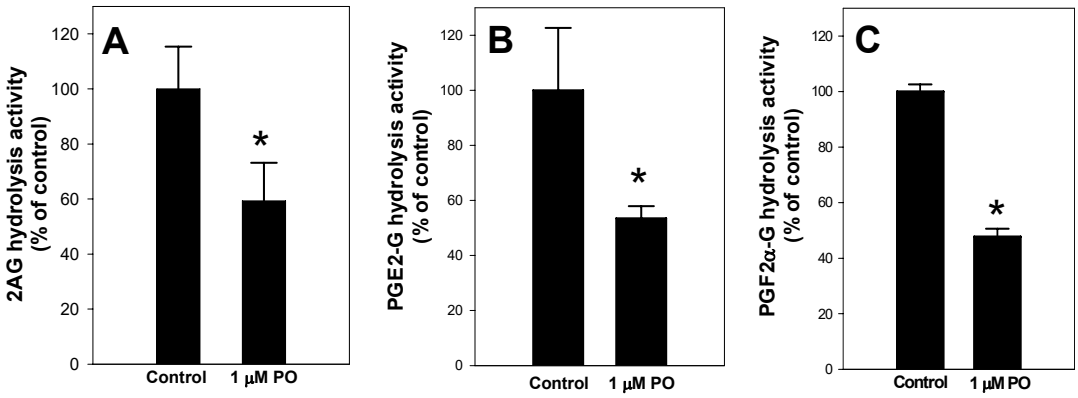


Figure 19. Paraoxon (PO) pretreatment of intact THP-1 monocytes reduces su sequent hydrolysis of exogenously added 2-AG (A), PGE_2-G (B), and $PGF_{2\alpha}$-G (C).

Cells were exposed to PO for 1 h followed by addition of lipid mediators to culture medium (and incubation for an additional 1 h). Data represent the mean ± SD of triplicate plates.

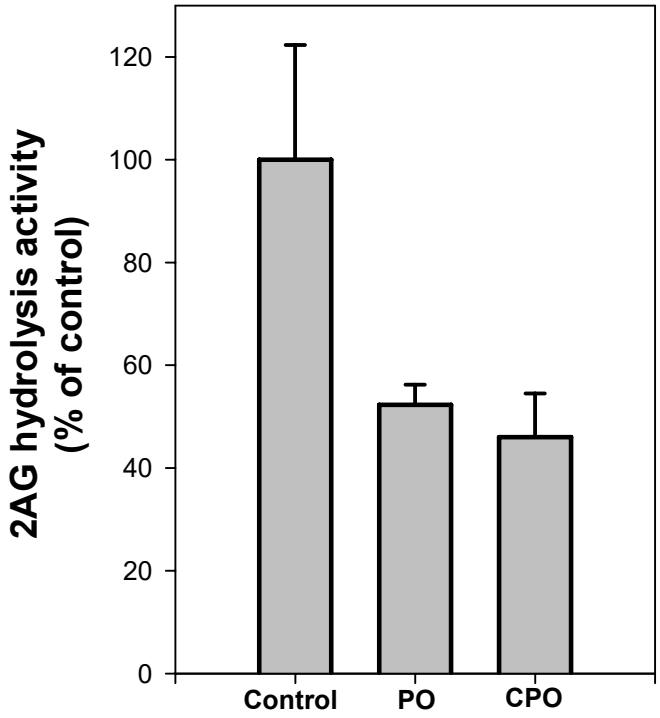


Figure 20. Inhibition of the 2-AG hydrolysis activity of THP1 monocyte lysates following treatment with PO or CPO.

THP1 cell lysate was pretreated with 1 μM PO or CPO for 30 min. Then 2-AG (10 μM) was added and the mixture incubated for an additional 30 min. The amount of arachidonic acid (AA) formed was assayed by LC-MS. Data represent the mean ± SD of triplicate reactions.

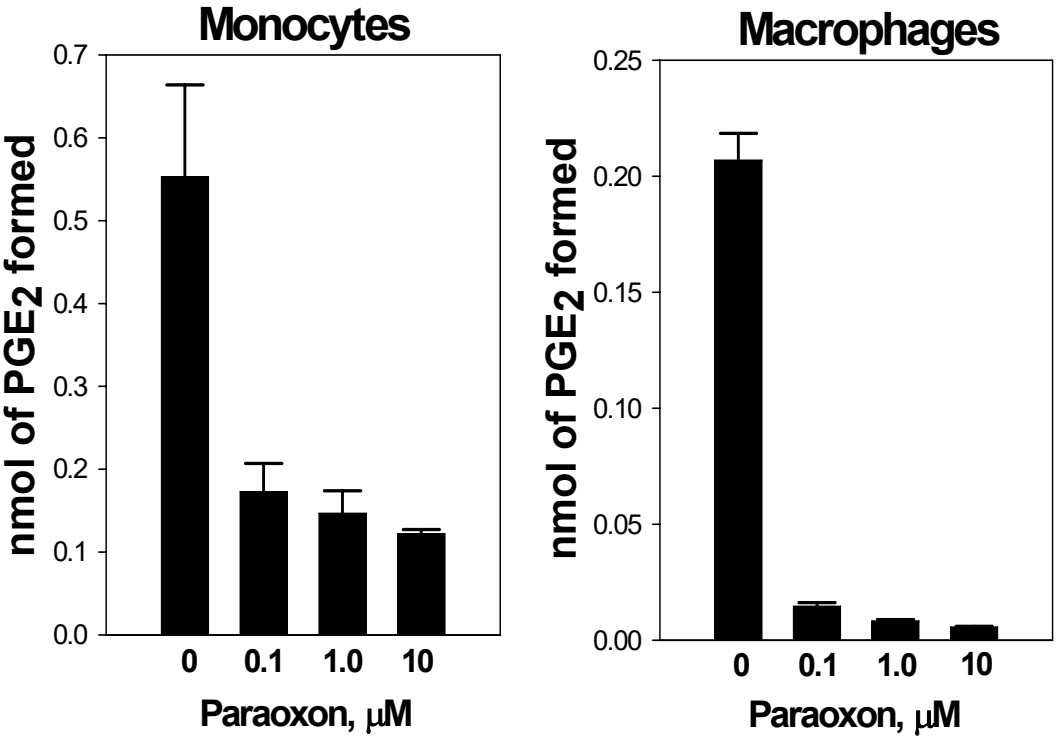


Figure 21. Intact THP-1 monocytes and macrophages were treated with the indicated concentrations of paraoxon.

After treatment with PO (24 h), cell lysates were prepared and incubated with synthetic PGE2-G (10 μM) for 15 min. Extracts were prepared and analyzed by LC-MS. Data represent the mean ± SD of duplicate or triplicate plates.

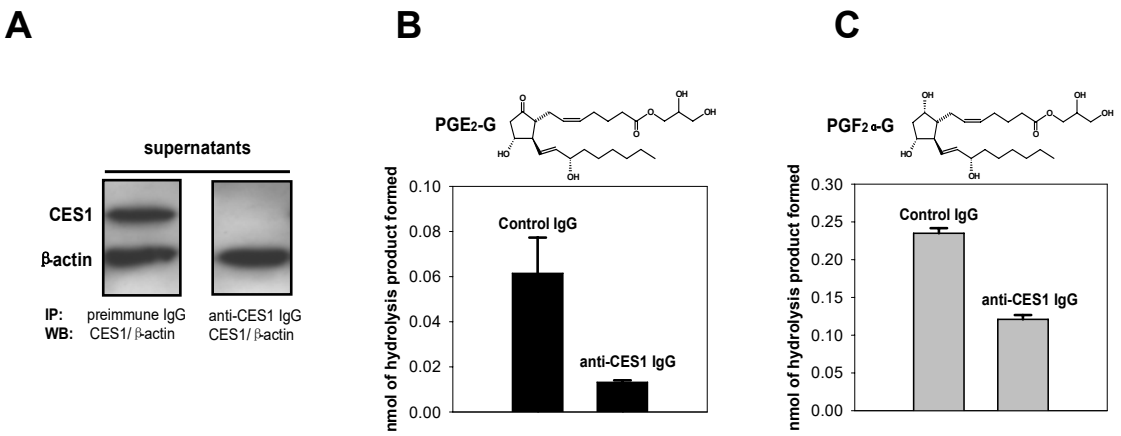


Figure 22. Hydrolysis of PGE_2-G and $PGF_{2\alpha}$-G by immunodepleted THP-1 lysates.

(A) Immunoprecipitation of CES1 from THP-1 monocyte lysates. Hydrolysis of PGE_2-G (B) and $PGF_{2\alpha}$-G (C) by immunodepleted THP-1 lysates following 30-min incubation with substrate. Data represent the mean ± SD of triplicate reactions.

CHAPTER V

CONCLUSIONS

The current study was conducted to examine interindividual variation of CES1 biochemical activity in humans, and to also determine if human CES have a role in degrading ester-containing lipid mediators, such as endocannabinoids and oxygenated endocannabinoid metabolites (PG-Gs).

It can be concluded that the abundance of CES1 protein expression in human liver does not predict its biochemical activity, which exhibits significant variation when probed with a CES1-selective substrate (bioresmethrin) or an activity-based probe (FP-biotin). Human interindividual variation in CES1 activities may be attributed to several mechanisms, including post-translational modifications, sequence polymorphisms, or protein-protein interaction. Because we found that arachidonic acid could inhibit CES1 activity, we measured the quantity of AA in the human livers. We did not observe a correlation between AA levels and CES1 biochemical activity. Therefore, it is concluded that AA does not account for the interindividual variation in CES1 biochemical activity that we observe.

Recombinant human CES1 and CES2 enzymes were found to efficiently hydrolyze 2-arachidonoylglycerol (2-AG) into arachidonic acid, and prostaglandin

glycerol esters (PG-Gs; PGE_2-G and $PGF_{2\alpha}$-G) into free prostaglandins, i.e. PGE_2 and $PGF_{2\alpha}$. The catalytic efficiencies of CES1 and CES2 toward 2-AG and PG-Gs are similar to or better than values published for recombinant rat MAGL and FAAH (Vila et al., 2007). Therefore, 2-AG and PG-Gs are novel endogenous substrates for CES1 and CES2 and add to the growing list of compounds that this enzyme metabolizes.

Using a small-molecule CES1-specific inhibitor (S-3030) or immunodepletion, it was found that CES1 may be an important hydrolytic enzyme for metabolizing 2-AG and PG-Gs in cultured human THP-1 monocytes. Furthermore, bioactive pesticide metabolites (such as paraoxon) can inhibit this biochemical activity of CES1 in THP-1 monocytes, resulting in reduced hydrolysis rates of exogenously added 2-AG or PG-Gs. These findings suggest that exposure of CES1- and/or CES2-expressing cells to bioactive OP metabolites may perturb the endocannabinoid and PG-G tone in tissues that are composed of these cells. This may have implications for diseases that macrophages have major pathological role in, e.g., atherosclerosis. Future studies will need to address one of the limitations of the current study, i.e. the reliance on supraphysiological concentrations of 2-AG and PG-Gs to study its metabolism in the cultured cells. Enhanced analytical methodology (e.g., LC-MS/MS) should help in this regard.

Finally, CES1 and MAGL are not the only enzymes in THP-1 monocyte cells that can hydrolyze 2-AG and PG-Gs. A lipase is present in this cell line that

is insensitive to the inhibitory effects of bioactive metabolites of chlorpyrifos and parathion. The identity of this lipase is currently unknown.

REFERENCES

Blankman JL, Simon GM, Cravatt BF. 2007 A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem Biol. 14(12): 1347-56.

Brash AR. 2001 Arachidonic acid as a bioactive molecule. Journal of Clinical Investigation. 107: 1339–1345

Brzezinski MR, Spink BJ, Dean RA, Berkman CE, Cashman JR, Bosron WF. 1997 Human Liver Carboxylesterase hCE-1: Binding Specificity for cocaine, heroin, and their metabolites and analogs. Drug Metabolism and Disposition. 25: 1089-1096.

Burston JJ, Sim-Selley LJ, Harloe JP, Mahadevan A, Razdan RK, Selley DE, Wiley JL. 2008 N-arachidonyl maleimide potentiates the pharmacological and biochemical effects of the endocannabinoid 2-arachidonylglycerol through inhibition of monoacylglycerol lipase. J Pharmacol Exp Ther. 327(2): 546-53.

Cashman J, Perroti B, Berkman C, Lin J. 1996 Pharmacokinetics and molecular detoxification. Environ Health Perspect 104: 23–40.

Clement JG., Erhardt N. 1990 Serum carboxylesterase activity in various strains of rats: sensitivity to inhibition by CBDP. Archives of Toxicology. 64: 414-416.

Crow JA, Herring KL, Xie S, Borazjani A, Potter PM, Ross MK. 2009 Inhibition of carboxylesterase activity of THP1 monocytes/macrophages and recombinant human carboxylesterase 1 by oxysterols and fatty acids. Biochim Biophys Acta. In press.

Crow JA, Middleton BL, Borazjani A, Hatfield MJ, Potter PM, and Ross MK. 2008 Inhibition of carboxylesterase 1 is associated with cholesteryl ester retention in human THP-1 monocyte/macrophages. Biochim Biophys Acta. 1781: 643-654.

David L, Guo XJ, Villard C, Moulin A, Puigserver A. 1998 Purification and molecular cloning of porcine intestinal glycerol- ester hydrolase Evidence for

its identity with carboxylesterase. European Journal of Biochemistry. 257: 142-148.

Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D. 2002 Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A. 99(16): 10819-24.

Duane WC, Javitt NB. 1999 27-Hydroxycholesterol: production rates innormal human subjects. The Journal of Lipid Research. 40: 1194-1199.

DuSell CD, McDonnell DP. 2008 27-Hydroxycholesterol: a potential endogenous regulator of estrogen receptor signaling. Trends in Pharmacological Sciences. 29: 510-514

Fu X, Menke JG, Chen Y, Zhou G, MacNaul KL, Wright SD, Sparrow CP, and Lund EG. 2001 27-Hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. Journal of Biological Chemistry. 276: 38378–38387.

Godin SJ, Crow JA, Scollon EJ, Hughes MF, DeVito MJ, Ross MK. 2007 Identification of rat and human cytochrome p450 isoforms and a rat serum esterase that metabolize the pyrethroid insecticides deltamethrin and esfenvalerate. Drug Metab Dispos. 35: 1664-1671.

Hosokawa M, Furihata T, Yaginuma Y, Yamamoto N, Watanabe N, Tsukada E, Ohhata Y, Kobayashi K, Satoh T, Chiba K. 2008 Structural Organization and Characterization of the Regulatory Element of the Human Carboxylesterase (CES1A1 and CES1A2) genes. Drug Metabolism and Pharmacokinetics. 23: 73–84.

Ileperuma NR, Marshall SDG., Squire CJ, Baker HM, Oakeshott JG., Russell RJ, Plummer KM, Newcomb RD, Baker EN. 2007 High-Resolution Crystal Structure of Plant Carboxylesterase AeCXE1, from Actinidia eriantha, and Its Complex with a High-Affinity Inhibitor Paraoxon. Biochemistry. 46: 1851-1859.

Imai T, Taketani M, Shii M, Hosokawa M, Chiba K. 2006 Substrate specificity of carboxylesterase isozymes and their contribution to hydrolase activity in the human liver and small intestine. Drug Metabolism and Disposition. 34: 1734–1741.

Kozak KR, Crews BC, Morrow JD, Wang LH, Ma YH, Weinander R, Jakobsson PJ, Marnett LJ. 2002 Metabolism of the Endocannabinoids, 2-Arachidonylglycerol and Anandamide, into Prostaglandin, Thromboxane, and

Prostacyclin Glycerol Esters and Ethanolamides. Journal of Biological Chemistry. 277: 44877-44885.

Liu Y, Patricelli MP, Cravatt BF. 1999 Activity-based protein profiling: the serine hydrolases. Proc Natl Acad Sci U S A. 96(26): 14694-9.

Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavón FJ, Serrano AM, Selley DE, Parsons LH, Lichtman AH, Cravatt BF. 2009 Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. Nat Chem Biol. 5(1): 37-44.

Miller AD, Scott DF, Chacko TL, Maxwell DM, Schlager JJ, Lanclos KD. 1999 Expression and Partial Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase. Protein Expression and Purification. 17: 16–250

Morton CL, Potter PM. 2000 Comparison of Escherichia coli, Saccharomyces cerevisiae, Pichia pastoris, Spodoptera frugiperda, and COS7 cells for recombinant gene expression. Application to a rabbit liver carboxylesterase. Mol Biotechnol. 16(3): 193-202.

Murase H, Sugihara A, Muro T, Shimada Y, Tominaga Y. 1991 Purification and Properties of Carboxylesterase from Ochrobactrum anthropi. Agric. Biol. Chem. 55: 2579-2584.

Redinbo MR, Potter PM. 2005 Mammalian carboxylesterases: from drug targets to protein therapeutics. Drug Discov Today 10: 313–325.

Ross MK, Borazjani A, Edwards CC and Potter PM. 2006. Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterases. Biochem. Pharmacol. 71: 657-669.

Ross MK, Pegram RA. 2004 In vitro biotransformation and genotoxicity of the drinking water disinfection byproduct bromodichloromethane: DNA binding mediated by glutathione transferase theta 1-1. Toxicol Appl Pharmacol. 195(2): 166-81.

Satoh T, Hosokawa M. 2006 Structure, function and regulation of carboxylesterases. Chemico-Biological Interactions. 162: 195–211.

Satoh T, Taylor P, Bosron WF, Sanghani P, Hosokawa M, Du BN. 2002 Current progress on esterases: from molecular structure to function. Drug Metabolism and Disposition. 30: 488–493.

Shimizu T. 2009 Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. Annu Rev Pharmacol Toxicol. 49: 123-50.

Sugamura K, Sugiyama S, Nozaki T, Matsuzawa Y, Izumiya Y, Miyata K, Nakayama M, Kaikita K, Obata T, Takeya M, Ogawa H. 2009 Activated endocannabinoid system in coronary artery disease and antiinflammatory effects of cannabinoid 1 receptor blockade on macrophages. Circulation. 119(1): 28-36.

Streit TM, Borazjani A, Lentz SE, Wierdl M, Potter PM, Gwaltney SR, Ross MK. 2008 Evaluation of the 'side door' in carboxylesterase-mediated catalysis and inhibition. Biol Chem. 389(2): 149-62.

Taketani M, Shii M, Ohura K, Ninomiya S, Imai T. 2007 Carboxylesterase in the liver and small intestine of experimental animals and human. Life Sciences. 81: 924-932.

Vila A, Rosengarth A, Piomelli D, Cravatt B, Marnett LJ. 2007 Hydrolysis of prostaglandin glycerol esters by the endocannabinoid-hydrolyzing enzymes, monoacylglycerol lipase and fatty acid amide hydrolase. Biochemistry. 46(33): 9578-85.

Wallace TJ, Ghosh S, Grogan W.M. 1999 Molecular Cloning and Expression of Rat Lung Carboxylesterase and Its Potential Role in the Detoxification of Organophosphorus Compounds. Cell Mol. Biol. 20: 1201–1208.

Zhao B, Song J, Chow WN, St Clair RW, Rudel LL, Ghosh S. 2007 Macrophage-specific transgenic expression of cholesteryl ester hydrolase significantly reduces atherosclerosis and lesion necrosis in Ldlr mice. J Clin Invest. 117(10): 2983-92.