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Carboxylesterases are uniquely expressed among tissues and regulated by nuclear hormone receptors in the mouse

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Running Title: Carboxylesterase mRNA distribution and regulation in mice

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Abbreviations: BAT, brown adipose tissue; CAR, constitutive androstane receptor (NR1I3); CES, carboxylesterase; C_q, real-time PCR cycle number at threshold used for quantification; CYP, cytochrome P450; FXR, farnesoid X receptor (NR1H4); IL-1β, interleukin-1β; LPS, lipopolysaccharide; LXR, liver X receptor (NR1H2 & H3); mpk, milligram per kilogram body weight; NHR, nuclear hormone receptor; PCN, pregnenolone-16α-carbonitrile; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome proliferator-activated receptor (NR1C1, C2, and C3); PXR, pregnane X receptor (NR1I2); qPCR, quantitative real-time PCR; RXR, retinoid X receptor (NR2B1, B2, and B3); TNF, tumor necrosis factor; WAT, white adipose tissue.

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ABSTRACT

Carboxylesterases (CES) are a well recognized, yet incompletely characterized family of proteins that catalyze neutral lipid hydrolysis. Some CES have well-defined roles in xenobiotic clearance, pharmacologic pro-drug activation, and narcotic detoxification. In addition, emerging evidence suggests other CES may have roles in lipid metabolism. Humans have six *CES* genes, while mice have 20 *Ces* genes grouped into five isoenzyme classes. Perhaps due to the high sequence similarity shared by the mouse *Ces* genes, the tissue-specific distribution of expression for these enzymes has not been fully addressed. Therefore, we performed studies to provide a comprehensive tissue distribution analysis of mouse *Ces* mRNAs. These data demonstrated that while the mouse *Ces* family 1 is highly expressed in liver and family 2 in intestine, many *Ces* genes have a wide and unique tissue distribution defined by relative mRNA levels. Furthermore, evaluating *Ces* gene expression in response to pharmacologic activation of lipid and xenobiotic-sensing nuclear hormone receptors (NHR) showed differential regulation. Finally, specific shifts in *Ces* gene expression were seen in peritoneal macrophages following LPS treatment and in a steatotic liver model induced by high-fat feeding, two model systems relevant to disease. Overall these data show that each mouse *Ces* gene has its own distinctive tissue expression pattern and suggest that some CES may have tissue-specific roles in lipid metabolism and xenobiotic clearance.

INTRODUCTION

Carboxylesterases (CES) comprise a family of proteins that catalyze neutral lipid hydrolysis (Hosokawa et al., 2007; Williams et al., 2010; Holmes and Cox, 2011). Substrate specificity among CES is often broad, overlapping, or yet to be identified leading to difficulty in performing comprehensive functional assays (Staudinger et al., 2010). Still, certain CES have long been recognized to play important roles in the biotransformation of ester- and amide-containing compounds to affect the detoxification and/or activation of a variety of xenobiotics, narcotics, and pharmacologic agents in liver and intestine (Satoh and Hosokawa, 1998; Satoh and Hosokawa, 2006). In addition, growing evidence suggests that some CES enzymes may contribute to aspects of lipid metabolism through triglyceride, cholesteryl ester, or retinyl ester hydrolysis (Schreiber et al., 2009; Parathath et al., 2011; Quiroga and Lehner, 2011b; Ghosh, 2012).

The CES family has been organized into five isoenzyme classes based on sequence similarity and gene structure (Hosokawa et al., 2007; Holmes et al., 2010b; Williams et al., 2010; Holmes and Cox, 2011). The six human *CES* genes, including one pseudogene, reside on chromosome 16; and the 20 mouse *Ces* genes, also including one pseudogene, are located on chromosome 8. The large number of *Ces* genes in rodents is believed to have arisen by tandem duplication. Thus, the sequence similarities of mouse *Ces* mRNA species and protein products are quite high, particularly within an isoenzyme class, which complicates the selective detection of *Ces* members *in vivo*.

With the recent description of a standardized nomenclature for mammalian carboxylesterases (Holmes et al., 2010b) and the advent of technologies that allow for quantitative and specific detection of closely related mRNA species (Valasek and Repa, 2005), we undertook a comprehensive survey of *Ces* mRNA expression in the mouse. Our goals were

to: firstly, determine the tissue distribution of mouse *Ces* mRNAs to identify organs beyond liver and intestine that express various *Ces* family members; secondly, evaluate the regulation of *Ces* gene expression by various nuclear hormone receptors (NHR) that serve as lipid and xenobiotic-sensing transcription factors, as well as pharmacologic targets (Chawla et al., 2001); and finally, to interrogate *Ces* expression in cell and organ systems relevant to disease, the macrophage and the steatotic liver. This *Ces* expression survey reveals novel tissues and regulation of family members, suggesting potential role(s) for these enzymes in lipid metabolism, xenobiotic clearance in extrahepatic tissues, as well as in the pharmacologic response to NHR activation by synthetic ligands.

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MATERIALS AND METHODS

Materials

The liver X receptor (LXR) agonist, T0901317 (T1317, (Repa et al., 2000)), was purchased from Cayman Chemical (Ann Arbor, MI); pregnane X receptor (PXR) agonist, pregnenolone-16α-carbonitrile (PCN, (Jones et al., 2000)) and the constitutive androstane receptor (CAR) agonist, 1,4-Bis-[2-(3,5-dichloro-pyridyloxy)]benzene, 3,3',5,5'-Tetrachloro-1,4-bis(pyridyloxy)benzyne (TCPOBOP, (Tzameli et al., 2000)), were obtained from Sigma-Aldrich Chemical (St. Louis, MO). Ligands for the peroxisome proliferator-activated receptors (PPARα-GW7647, (Brown et al., 2001); PPARβ-GW0742, (Sznaidman et al., 2003) ; and PPARγ-GW7845, (Henke et al., 1998)) and FXR (GW4064, (Maloney et al., 2000)) were provided by Timothy M. Willson (Glaxo SmithKline), and the retinoid X receptor (RXR) agonist LG268 (Mukherjee et al., 1997) from Richard A. Heyman (Aragon Pharmaceuticals).

Animals and treatments

Mice were housed in a temperature-controlled environment with 12h light/dark cycles (light: 06:00-18:00) and allowed free access to water and a cereal-based rodent diet (#7001; Teklad Diets, Madison, WI). For establishing the tissue distribution of *Ces* mRNAs, three male C57Bl/6 mice were euthanized (at 10:00, 4h after lights on) by exsanguination under deep anesthesia, and tissues were harvested. For evaluating NHR regulation of *Ces* gene expression, male A129/SvJ mice received ligands by oral gavage, as this strain of mice has been previously used for similar studies where drug doses were established (Cha and Repa, 2007). Agonists were suspended in 1% w/v methylcellulose and 1% v/v Tween-80 (vehicle) to deliver the following quantities of ligand in each dose using 10 µl/g body weight: LG268, 30 mg/kg body weight (mpk); GW7647, 10 mpk; GW0742, 10 mpk; GW7845, 20 mpk; T1317, 30 mpk; GW4064, 100 mpk; PCN, 50 mpk, and TCPOBOP, 3 mpk. For gavage-dosing, treatments were administered at

the beginning of the dark cycle (at 18:00) and again 12h later at the beginning of the light cycle (at 06:00), at which point food was removed. After 4h (at 10:00), mice were euthanized, and tissues harvested. For the high-fat diet study, male C57BL/6 mice were fed either high-fat diet containing 58 kcal% fat provided by soybean and coconut oils (#D12331; Research Diets, New Brunswick, NJ), or a cereal-based rodent diet (#7001, Teklad) for 16 weeks before livers were harvested at 10:00.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Cell culture

All cells were cultured in a 37°C humidified incubator with 5% CO_2.

Cell lines: The adenoma-derived glucagonoma cell line αTC1-clone 9 ([CRL-2350], (Powers et al., 1990)) was obtained from American Type Culture Collection (Manassas, VA). The insulin-secreting MIN6 cell line, passage #24 (Miyazaki et al., 1990) was kindly provided by Melanie Cobb (UT Southwestern). αTC1 cells were cultured in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 3 g/L glucose with 10% heat-inactivated dialyzed FBS, further supplemented with 15 mM HEPES, 0.1 mM non-essential amino acids and 0.02% BSA. MIN6 cells were maintained in DMEM (4.5g/L glucose) with 2 mM L-glutamine, 1 mM sodium pyruvate and 10% heat-inactivated FBS.

Islets: Mouse pancreatic islets were prepared as previously described (Chuang et al., 2008). Briefly, the pancreata from three C57Bl/6 male mice were perfused and digested with liberase R1 (Roche, Indianapolis, IN). Islets were then isolated using Ficoll gradient

centrifugation and hand-selection under a stereomicroscope for transfer to RPMI 1640 medium (11.1 mM glucose) supplemented with 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Islets were allowed to recover overnight before RNA was isolated.

Macrophages: Male A129/SvJ mice received an intraperitoneal injection of 1 ml 3%-thioglycollate (autoclaved and aged for 3 months) to elicit macrophages. Three days later, mice were euthanized and macrophages were withdrawn by sterile lavage using ice-cold saline. Cells were collected by gentle centrifugation and plated at $1x10^5$ cells/cm^2 in high-glucose DMEM containing 10% heat-inactivated FBS and 100 IU/ml penicillin/100 µg/ml streptomycin. After 6h, cells were washed with sterile PBS and provided fresh media containing saline (vehicle, 0.1% v/v) or 100 ng/ml lipopolysaccharide (LPS, Sigma). Cells were harvested 4h later for RNA isolation.

Preparation of samples for RNA measurements

Mice were anesthetized and exsanguinated via the inferior vena cava. Small intestines were removed, flushed with ice-cold saline, and cut into three sections of equal length which we denote as duodenum, jejunum, and ileum. The sections were slit lengthwise, and the mucosae were obtained by gentle scraping. Intestinal mucosa, liver, adrenal glands, brain, epididymal white adipose tissue, intrascapular brown adipose tissue, kidneys, lung, skeletal muscle (quadriceps), and spleen samples were flash-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from tissue samples, cultured cells, and islets using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) as previously described (Kurrasch et al., 2004). RNA concentration was determined by absorbance at 260 nm, and RNA quality by the 260/280 ratio.

Quantitative real-time PCR

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Quantitative real-time PCR (qPCR) was performed using an Applied Biosystems 7900HT sequence detection system as described (Kurrasch et al., 2004; Valasek and Repa, 2005). Briefly, total RNA was treated with DNase I (RNase-free; Roche Molecular Biochemicals) and reverse-transcribed with random hexamers using SuperScript II (Invitrogen) to generate cDNA. Primers for each gene were designed using a variety of primer design algorithms: DLux (Invitrogen, https://orf.invitrogen.com/lux/); PrimerBlast (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast) and Integrated DNA Technology (http://www.idtdna.com/scitools/applications/RealTimePCR/default.aspx) to ensure that for each target: primers spanned an intron; primers did not anneal to unanticipated off-site cDNA or genomic sequences, and primers exhibited maximal 3' mismatch for closely related CES family members to achieve target specificity. All primer pairs were validated by: analysis of template titration (acceptable primers exhibited slopes of -3.3 ± 0.1 for plot of C_q vs log[cDNA, ng], (Bustin et al., 2009)); appearance of a single peak upon a graded temperature dissociation analysis; and the absence of PCR product upon omission of template cDNA. Of note, no discernible PCR product was generated using three different primer sets designed for *Ces1b*, using template cDNA from liver, intestine, kidney, and a universal RNA mixture. It is likely that *Ces1b* mRNA is expressed at levels too low to detect by qPCR or expressed in minor cell or tissue types. Finally, while multiple transcript isoforms, generated by alternative promoter usage and/or alternative splicing of exons, have been described for human *CES* genes, this is not yet the case for mouse *Ces* genes, thus our qPCR primer designs are consistent with the current GenBank reference sources, and all primer sequences and gene annotations are provided in Supplemental Table 1.

Each qRT-PCR was analyzed in duplicate and contained in a final volume of 10 µl: 25 ng of cDNA, each primer at 150 nM, and 5 µl of 2x SYBR Green PCR Master Mix (Applied

Biosystems). Results were evaluated by the comparative cycle number at threshold method (Schmittgen and Livak, 2008) using cyclophilin as the invariant reference gene (Dheda et al., 2004; Kosir et al., 2010).

Analysis of data

Data are reported as means ± SEM for the number of animals per tissue and/or treatment group, as specified in each figure legend. GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA) was used to perform all statistical analyses. For the multi-agonist study, statistical differences were determined by one-way ANOVA with Dunnett's *post hoc* analysis, which compares each treatment group to the vehicle-treated control group (significantly different groups denoted by an asterisk *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). A Student's *t*-test was used for comparison of only 2 groups.

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RESULTS

The CES1 family is highly expressed in liver and the CES2 family in intestine, but these and other CES members are present in a wide variety of tissues and cells in the mouse.

Numerous reports have appeared describing the distribution, regulation and function of various carboxylesterases in the mouse model (Poole et al., 2001; Holmes et al., 2009; Xu et al., 2009; Holmes et al., 2010a; Staudinger et al., 2010; Zhang et al., 2012). However, with the confusion regarding nomenclature and ortholog assignment, and the nonselectivity of some oligonucleotide and antibody probes, the reliability of these findings has been uncertain. With the standardization of *Ces* nomenclature (Holmes et al., 2010b) and the target specificity available using quantitative real-time PCR (qPCR), we have now performed a comprehensive survey of *Ces* expression in the mouse. The utility of our qPCR method is readily apparent by the distinct expression patterns of *Ces* mRNAs, thus confirming the specificity of primers. In addition, the rank order of *Ces* mRNA levels in liver tissue observed in our studies is consistent with recently published findings for 11 *Ces* members using a branched-DNA signal amplification assay (Zhang et al., 2012). Hence our qPCR method provides a reliable means to determine steady-state levels of *Ces* mRNA in the mouse.

The mouse *Ces1* family consists of eight genes *Ces1a*-*Ces1h* (Fig. 1) located in tandem on chromosome 8 (between coordinates 95,544,116 – 95,903,624). Each mouse *Ces1* gene contains 13-14 exons and encodes a protein with a predicted mass of 62 kDa. The *Ces1* family has been reported to be expressed predominantly in liver (reviewed in (Holmes et al., 2010b)), and indeed all detected *Ces1* members were present in liver; however, we noted four important points. Firstly, there was no detectable expression of *Ces1b* mRNA in any tissue tested, including liver, despite our efforts to identify primer sets and tissues that would yield a qPCR

product by this method. Secondly, *Ces1d* mRNA was detectable in liver, but was far more abundant in brown (BAT, denoted as "X" in Fig. 1) and white adipose tissue (WAT, W in Fig. 1). Thirdly, *Ces1f* mRNA levels were greatest in kidney, a tissue that expressed high levels of many *Ces* members. Finally, while the highest mRNA levels for *Ces1h* are found in mouse lung, it should be noted that the quantification cycle (C_q, (Bustin et al., 2009)) observed for this PCR product is nearly at the limit of detection. Thereby this mRNA species is found only at very low levels in all mouse tissues in which it was expressed. Thus, in line with previous studies describing the *Ces1* family members as the hepatic carboxylesterases, four of the seven detectable mRNAs (*Ces1a, Ces1c, Ces1e, and Ces1g*) were most highly expressed in liver compared to 15 other mouse tissues and cell types.

The mouse *Ces2* family contains eight members (*Ces2a-Ces2h*) with one, *Ces2d*, identified as a pseudogene (excluded in our survey). In line with previous studies which have shown that family 2 encodes the intestinal carboxylesterases (reviewed in (Holmes et al., 2010b)), almost all the *Ces2* members were most highly expressed in segments of the small intestine (Fig. 2). The only exception was *Ces2h*, which exhibited the highest mRNA levels in kidney, followed closely by the small intestine. For all *Ces2* members, there was a cephalocaudal distribution of mRNA with highest levels in the proximal third (duodenum) of the small intestine, and decreasing amounts throughout the rest of the small bowel and into the colon. Of note, although *Ces2* mRNA levels are highest in the intestine, there is also relatively high expression in other organs including liver (*Ces2a, 2e*), kidney (*Ces2c*), and spleen (*Ces2g*). *Ces2f* mRNA levels were extremely low in all tissues tested.

The mouse *Ces3* family includes two genes, *Ces3a* and *Ces3b*, which are almost exclusively expressed in liver (Fig. 3). **Families 4 and 5** have only one member each, *Ces4a* and

Ces5a. *Ces4a* mRNA levels are highest in liver, and *Ces5a* expression is evident in WAT, brain, and the glucagonoma alpha-cell line (αTC1).

The rank order of *Ces* mRNA levels within tissues reveals that members outside of family 1 are likely to play an important role in liver, and kidney and brain exhibit unique expression patterns of *Ces* mRNA species.

Our experimental strategy allowed for a rank order determination of mRNA levels for *Ces* members within a given tissue (Fig. 4). The PCR primers were designed to provide equivalent PCR amplification efficiency for all *Ces* targets and showed no product formation in the absence of template cDNA. Therefore, as the same samples were used for all *Ces* mRNA analyses, we could compare the relative expression of all 18 *Ces* members to one another in a given tissue or cell type. Viewing the data in this rank order format for liver reveals that in addition to the "liver" CES1 family of enzymes, CES2A, CES3A, and CES3B proteins may play some role in hepatic lipid metabolism and/or xenobiotic response. The "intestine" *Ces2* family are by far the most abundant *Ces* members expressed in the duodenum (*Ces2c* >> *Ces2a*=*Ces2e*). In lung, *Ces1d* mRNA levels are so abundant (C_q=17.2) that other *Ces* members (*Ces1e*, *1f*, and *2g*), which exhibit significant (C_q <24) expression, and *Ces1h*, which is found nearly exclusively in this tissue, are barely evident. The kidney and brain express a unique complement of "liver" *Ces1* (*Ces1d* and *1f*) and "intestine" *Ces*2 (*Ces2c*) mRNA species, and brain is the only tissue among those we examined that exhibits appreciable relative levels of *Ces5a* mRNA, which encodes a secreted carboxylesterase member also known as cauxin, previously identified in urine and epididymal fluid (Ecroyd et al., 2006; Miyazaki et al., 2006). Our results for *Ces5a* mRNA distribution are consistent with those reported for ram tissues, in which expression was not observed in kidney (Ecroyd et al., 2006), and to our knowledge our findings are the first to

demonstrate appreciable *Ces5a* mRNA expression in brain, which was not evaluated in previous studies. For all other mouse tissues tested, except cells of the endocrine pancreas which do not appear to abundantly express any carboxylesterase mRNA, the rank order data resemble those of lung and white adipose tissue, with other CES members dwarfed by *Ces1d* expression (data not shown).

Short-term administration of synthetic agonists for nuclear hormone receptors results in differential expression of *Ces* family members.

A subset of the NHR superfamily of ligand-activated transcription factors has been characterized as lipid or xenobiotic sensors (Chawla et al., 2001). For these NHRs (LXRs, FXR, PPARs, PXR and CAR), ligand-activation induces the expression of genes encoding transporters, enzymes and binding proteins to modulate the intracellular levels for a receptor's respective ligand. To test whether *Ces* mRNA expression is subject to this regulation, and perhaps reveal novel lipid metabolic pathway(s) affected by *Ces* gene products, we evaluated *Ces* expression in a variety of tissues from mice treated with NHR agonists. Potent synthetic agonists which have been developed for pharmacologic activation of specific NHRs were administered to mice by oral gavage twice, 16h and 4h prior to harvesting tissues, to allow sufficient time for direct gene activation or repression by NHRs. The efficacy of this dosing regimen was confirmed by the measurement of known hepatic target genes for these NHRs (Fig. 5A). Cholesterol 7α-hydroxylase (*Cyp7a1*) mRNA levels were increased 9.7-fold by an LXR agonist and decreased by ligands for RXR (to 1%), FXR (to 7%), and PXR (to 22%), changes consistent with previous reports (Repa et al., 2000). The PPAR target gene, cytochrome P450 4A14 (*Cyp4a14*, (Patsouris et al., 2006)) exhibited greater hepatic mRNA levels in mice treated with a PPARα agonist (GW7647, 34-fold), a PPARβ ligand (GW0742, 10.5-fold) and an RXR drug (LG268, 11.3-

fold). Finally, administration of ligands for the xenobiotic receptors, PXR and CAR, resulted in enhanced expression of the drug-metabolizing enzyme, cytochrome P450 3A11 (5.2-fold for PXR, 2.7-fold for CAR). Thus, this relatively short-term (16h) dosing method delivered sufficient NHR agonists to affect gene transcription.

In liver, we observed numerous changes in *Ces* mRNA levels following administration of NHR ligands to mice (Fig. 5B). PPARα activation resulted in increased expression of *Ces1d* (5.2-fold), *Ces1e* (4.7-fold), *Ces1f* (3.4-fold), *Ces2c* (3.3-fold), and *Ces2e* (1.7-fold). PPARβ activation significantly increased the expression of *Ces1e* (2.3-fold), and *Ces2e* (1.7-fold). Administration of agonists for LXR, PXR, and CAR all resulted in increased hepatic mRNA levels of *Ces2a* (3-, 7.6- and 4.8-fold respectively), while PXR activation significantly decreased expression of *Ces3a* (to 76%). *Ces2c* was the most responsive hepatic carboxylesterase to NHR activation, as its expression was increased by agonists for RXR (4.8-fold), PPARα (3.3-fold), LXR (2.4-fold), and CAR (2.9-fold). *Ces4a* expression was significantly increased upon RXR (3.1-fold) activation alone. Of note, the hepatic expression of *Ces1d*, *Ces1e*, and *Ces1f* due to NHR activation had very similar patterns (Fig. 5B), even though they have unique tissue distribution profiles (Fig. 1). In mucosa obtained from the proximal third of the small intestine (duodenum), the mRNA levels of two *Ces* members were altered by NHR activation (Fig. 5C). Duodenal *Ces2a* mRNA levels were increased by agonists for LXR (2.4-fold), FXR (4.3-fold), PXR (7.1-fold), and CAR (2.1-fold); whereas *Ces2c* was significantly upregulated by only PXR activation (1.6-fold). Finally, in the lung, *Ces* mRNA levels were largely unaffected by NHR ligand treatment, with only a modest, but significant, increase observed for *Ces1e* mRNA levels. It is of interest that certain genes such as *Ces2c* and *Ces1d* respond differently to NHR activation in different organs, suggesting there is tissue-specific regulation of these carboxylesterases.

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Differential expression of *Ces* in LPS-activated macrophages and in steatotic livers of high-fat fed mice.

We extended this *Ces* expression survey to cells and tissues associated with disease: the activated macrophage (atherosclerosis and inflammatory disease) and the steatotic liver (insulin resistance and the metabolic syndrome). As these disease-associated conditions have also been the subject of transcriptomic and lipidomic analyses (Joseph et al., 2003; Welch et al., 2003; Barish et al., 2005; Dennis et al., 2010; Fu et al., 2011), the concomitant regulation of *Ces* members may reveal pathways of lipid metabolism affected by these enzymes.

A carboxylesterase cloned from human monocytes (*CES1*) has been characterized and found to promote cholesteryl ester hydrolysis when overexpressed in macrophages or mice (Ghosh, 2000; Zhao et al., 2007; Ghosh, 2012). However, a functional ortholog of this enzyme in the mouse macrophage has not yet been identified (reviewed in (Quiroga and Lehner, 2011b)). More recently, human *CES3* has also been implicated in cholesteryl ester hydrolysis in macrophages (Zhao et al., 2012), and the expression of this CES member appears to be dependent on the abundance of CES1. Again, however, it is unclear which mouse *Ces* member may impart this CES3 function in macrophages. Thus we evaluated the expression of all mouse *Ces* mRNAs in primary mouse macrophages (Fig. 6A). Thioglycollate-elicited peritoneal macrophages were harvested from mice and then treated in culture for 4h with either vehicle (saline) or lipopolysaccharide (LPS) to activate an inflammatory response as demonstrated by an increase in mRNA levels for the cytokines, interleukin 1β (*Il1β*) and tumor necrosis factor (*Tnf*) (Fig. 6A inset). *Ces1c*, *Ces2f*, and *Ces2g* were the most abundant carboxylesterase mRNA species in unstimulated mouse macrophages. After LPS treatment, a significant decrease in the expression of *Ces1c* and a nearly significant decline in *Ces2f* mRNA levels (p=0.06) occurred, making *Ces2g* the most plentiful carboxylesterase in the activated macrophage. It should be

noted that the mRNA level for the most abundant macrophage carboxylesterase, *Ces1c* (qPCR C_q=28.8) represents less than 1% of the mRNA level for *Ces1c* observed in liver (qPCR C_q= 16.6), which suggests a low abundance of these enzymes in the mouse macrophage. Future studies will be required to ascertain the functional contribution of mouse *Ces* members to macrophage lipid biology, in analogy to the reports regarding the roles of *CES1* and *CES3* in human macrophage cholesterol homeostasis (Ghosh, 2000; Zhao et al., 2007; Ghosh, 2011; Zhao et al., 2012).

Finally, to complement our survey of *Ces* gene regulation by lipid-sensing transcription factors, we elected to evaluate *Ces* mRNA levels in a steatotic liver model induced by feeding a high-fat diet. Male C57Bl/6 mice were fed *ad libitum* a diet containing 58 kcal% fat (soybean and coconut oils) for 16 weeks. Compared to mice fed a standard chow diet, the mice fed high-fat had greater body weight (1.67-fold), increased liver weight (1.31-fold), and impaired glucose tolerance (increased fasting glucose, and more profound glucose excursion following an oral glucose challenge) (data not shown). The "liver" *Ces1* family showed very little change in mRNA levels, except the most abundant member, *Ces1c* (see Fig. 4), for which mRNA levels were reduced about 20% by high-fat feeding. The steatotic livers of these high-fat fed mice also exhibited increased *Ces2e* and *Ces2g* mRNA, while *Ces2a* and *Ces4a* mRNA levels were decreased.

DISCUSSION

To our knowledge this is the first comprehensive analysis of *Ces* expression in the mouse. This project was largely instigated by the recent report recommending a standardized nomenclature for *Ces* genes (Holmes et al., 2010b), and the fact that these genes repeatedly appear in microarray data, where it is often unclear which *Ces* member(s) is represented. We elected to perform this survey in mice, as these are widely used subjects in lipid and xenobiotic research, for which a large collection of genetic, dietary, and drug-induced models of lipid-related disease are available. In addition, the mouse has 20 *Ces* genes, the largest number of any mammalian species reported to date (Williams et al., 2010), thus making the selectivity and sensitivity of our qPCR method especially appropriate.

The first important and quite surprising finding was that these 20 *Ces* members exhibit very distinct expression patterns. It is thought that the 20 mouse *Ces* genes arose through tandem duplication, and indeed, they share remarkable sequence similarity and gene structure. They even reside in a cluster on mouse chromosome 8, which would suggest that they might share enhancer elements and regulatory sequences to dictate their expression levels, as seen for hepatic *Ces1d*, *Ces1e*, and *Ces1f* due to NHR activation (Fig. 5). However, while there are some general patterns (*Ces1*s enriched in liver, *Ces2*s abundant in intestine), each *Ces* member is expressed in a unique set of tissues (Figs. 1-4), and many show selective changes in gene expression by drug (Fig. 5, Fig. 6A) or diet (Fig. 6B) treatment. These data provide valuable insight regarding potential xenobiotic activity of CES in organs other than the intestine and liver. While major questions still remain about these enzymes including the identification of substrates, which remains difficult, and the localization of activity, this report will inform future studies.

The second finding is that the mRNA levels of several *Ces* members are regulated by the lipid-sensing nuclear hormone receptors. The role of carboxylesterases in drug metabolism has

been widely appreciated, and previous work from the Klasseen and Staudinger groups has convincingly shown that the xenobiotic nuclear hormone receptors PXR and CAR increase expression of *Ces2a* (previously *Ces6*) and *Ces2c* (Xu et al., 2009; Staudinger et al., 2010; Zhang et al., 2012). Our work confirms these findings (Fig. 5) and extends these analyses to reveal that NHRs traditionally associated with fatty acid and/or sterol regulation can likewise regulate the expression of *Ces* mRNAs:

Bile acids/FXR. A previous report suggested that modifying bile acid homeostasis in mice by depletion using cholestyramine or supplementation with sodium cholate increased hepatic levels of *Ces1g* mRNA (previously called ES-x, (Ellinghaus et al., 1998)). Our specific, synthetic agonist, GW4064, for the bile acid nuclear receptor, FXR, did not recapitulate these findings in liver, nor were changes in *Ces1g* mRNA observed in mouse intestine (data not shown), suggesting that alternate bile acid-responsive, non-FXR mechanisms may be responsible for differential *Ces1g* expression.

Fatty acids & fibrates/PPARs. Early studies in mice and rats demonstrated that certain drugs that enhanced hepatic peroxisome proliferation also caused an increase in microsomal carboxylesterase activity (Mentlein et al., 1986; Ashour et al., 1987; Hosokawa and Satoh, 1993; Parker et al., 1996). In particular, treating rodents with the PPARα ligands clofibrate, WY14,653, or perfluorinated fatty acids resulted in increased CES enzyme activity of 5-6 subclasses defined by substrate specificity. Most recently two reports have demonstrated that fibrate treatment of C57/Bl6 mice for 5 days to 2 weeks does not upregulate hepatic *Ces1d* mRNA levels (Dolinsky et al., 2003; Zhang et al., 2012). These recent findings differ from our results showing that administration of the potent, specific PPARα agonist GW7647 to A129/SvJ mice is associated with increased expression of *Ces1d*, *Ces1e*, *Ces1f*, *Ces2c* and *Ces2e* mRNA in liver. These differing findings could be due to drug specificity (Thomas et al., 2002); duration of

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dosing, and/or mouse strain (although our studies were not designed and optimized for direct comparison of strains, the C_q relative to reference cyclophilin gene expression suggests that C57Bl/6 mice exhibit higher basal *Ces1d* mRNA levels than A129/SvJ, which may preclude them from exhibiting robust upregulation of this Ces family member).

Oxysterols/LXR. Treatment with ligands for the "sterol-sensing" LXRs resulted in increased expression of hepatic and intestinal *Ces2a* and *Ces2c*. In addition, a synthetic ligand for the common heterodimer partner, RXR, caused a robust increase in *Ces2c* and *Ces4a* mRNA levels. These results suggest either a combinatorial effect of RXR with other regulators (PPARα, LXR in the case of *Ces2c*), or a novel transcriptional mechanism involving RXR homodimers and/or other RXR heterodimer partners not interrogated in our experiments (such as RARs, VDR, or NGFIB). It should be noted that T1317, the LXR agonist used in these studies, has been shown to function as a potent ligand for human PXR (Xue et al., 2007), and to exhibit some activity towards mouse PXR and FXR (Repa et al., 2000; Mitro et al., 2007). While we did not detect a significant increase in the mRNA levels of *Cyp3a11*, the surrogate marker of PXR activation used to define drug specificity and potency in our studies (Fig. 5A), we can not rule out the possibility that PXR mediates some of the effects of T1317 in regulating hepatic *Ces2a* expression.

The mammalian carboxylesterases have become enzymes of increasing interest, particularly in regards to potential activities as cholesteryl ester and/or triacylglyceride hydrolases (Quiroga and Lehner, 2011a; Ghosh, 2012; Zechner et al., 2012). Recombinant mouse *Ces1e* (called Es22) enzyme shows robust retinyl ester hydrolysis activity and modest, albeit significant, activity as a cholesteryl ester hydrolase (Schreiber et al., 2009). It has been noted that several tissues exhibit triglyceride hydrolysis activity that cannot be accounted for by known lipases (e.g. ATGL, HSL, and others), thus supporting the existence of additional neutral

lipases (Zechner et al., 2012). Mouse *Ces1d* (also called TGH or Ces3) has been identified as a triglyceride hydrolase (Dolinsky et al., 2001), and mice lacking this enzyme show dramatic changes in energy expenditure, plasma lipoprotein profiles and hepatic steatosis (Wei et al., 2010). Thus a number of *Ces* members have been implicated in regulation of lipid hydrolysis, however except for *Ces1d*, it is not clear if or which mouse *Ces* gene(s) may show similar activities. This report reveals many other *Ces* genes which may be involved in these processes, based solely on evidence from gene expression changes due to lipid-sensing transcription factors, and disease models. Acute activation of fatty acid-sensing transcription factors (RXRs, PPARs see Fig. 5) results in increased hepatic RNA levels of *Ces1*members (*1d, 1e, and 1f*), although chronic high-fat feeding of mice does not result in altered expression of these genes in the steatotic liver (Fig. 6B). The *Ces2* members exhibit both acute (*Ces2a, Ces2c, and Ces2e)* effects due to short-term administration of ligands to the lipid-sensing nuclear hormone receptors, and differential mRNA levels in the steatotic liver (*Ces2e* and *Ces2g*). Finally, two *Ces* genes exhibited altered expression in LPS-treated mouse macrophages (*Ces1c* and *Ces2f*), suggesting that these carboxylesterases may play a role in the lipid biology of this important cell type, as previously reported for other *CES* members in human macrophages (Ghosh, 2000; Zhao et al., 2007; Ghosh, 2011; Zhao et al., 2012).

While it is clear that there are many post-transcriptional levels of regulation which limit the interpretation of mRNA expression data, this survey offers valuable information to guide future studies of the CES family of enzymes. Given the current lack of CES-selective substrates and/or antibodies for the 19 mouse enzymes, a comprehensive analysis of protein abundance or activity is not yet possible. Therefore this description of mouse distribution and regulation of *Ces* mRNA expression provides a framework for future research into the pharmacologic

response, and extrahepatic xenobiotic clearance capacity of the highly similar, yet variably regulated carboxylesterases.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Jones, Repa

Conducted experiments: Jones, Taylor, Tong

Performed data analysis: Jones, Taylor, Tong

Wrote or contributed to the writing of the manuscript: Jones, Repa

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FOOTNOTES

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b) This work has not been previously presented.

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FIGURE LEGENDS

Figure 1. *Tissue Distribution of the Carboxylesterases: Family 1.* The relative mRNA levels are depicted for mouse liver (L), duodenum (D), jejunum (J), ileum (I), colon (C), adrenal (A), islet (E),α-cell line (αTC1, α), and β-cell line (MIN6, β), brain (B), epididymal white adipose tissue (WAT, W), intrascapular brown adipose tissue (BAT, X), kidney (K), lung (U), muscle (M), and spleen (S). Tissues were obtained from adult (3-5 months of age) C57Bl/6 mice that were fed *ad libitum* a standard low-fat rodent diet. Individual mRNA values were calculated relative to cyclophilin and the mean values were arithmetically adjusted to depict the highest-expressing tissue as a unit of 100. Values represent the means ± SEM of three independent samples for each tissue or cell line. Note, that as these data are portrayed, comparisons can only be made between the different tissues for a single *Ces* mRNA family member, not between the various *Ces* mRNA species (see Fig. 4 for this comparison). The average cycle-at-threshold (C_q) value used for quantification is provided for the tissue showing the most abundant mRNA level for each *Ces*.

Figure 2. *Tissue Distribution of the Carboxylesterases: Family 2.* Refer to the legend of Fig.1 for details.

Figure 3. *Tissue Distribution of the Carboxylesterases: Families 3, 4 and 5.* Refer to the legend of Fig.1 for details.

Figure 4. *Comparative Expression Levels of mRNA for Carboxylesterases of Liver, Duodenum, Kidney and Lung.* The relative mRNA levels of all *Ces* family members are provided for each tissue. Individual values were calculated relative to cyclophilin and the means were

arithmetically adjusted to depict the highest-expressed *Ces* mRNA species for each tissue as a unit of 100. Values represent the means ± SEM of three independent samples for each tissue.

Figure 5. *Regulation of Carboxylesterase mRNA Levels by Nuclear Hormone Receptor Agonists.* Relative mRNA levels are depicted for adult (3-5 months of age) A129/SvJ mouse liver, duodenum and lung. Tissues were harvested from mice that had received two doses of nuclear receptor agonist by oral gavage (16h and 4h before tissue collection). Each dose provided agonist of a given nuclear hormone receptor as follows: Vehicle (1% w/v methylcellulose, 1% v/v tween-80), 30 mg/kg body weight, "mpk" LG268 (RXR), 10 mpk GW7647 (PPARα), 10 mpk GW0742 (PPARβ), 20 mpk GW7845 (PPARγ), 30 mpk T1317 (LXR), 100 mpk GW4064 (FXR), 50 mpk PCN (PXR), and 3 mpk TCPOBOP (CAR). Individual values were calculated relative to cyclophilin and the mean values were arithmetically adjusted to depict the vehicle-treated group at a unit of 1. **A.** Changes in the hepatic expression of selected cytochrome P450 enzymes confirm the efficacy of this dosing regimen. *Ces* mRNA levels are shown for liver (**B**), duodenum (**C**), and lung (**D).** Values reflect the means ± SEM (n=4). *, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$ compared with the vehicle-treated group (white bar), as determined by one-way ANOVA with Dunnett's post-hoc comparison.

Figure 6. *Comparative Expression of Carboxylesterases in Thioglycollate-elicited Mouse Peritoneal Macrophages and in the Livers of High Fat Diet Fed Mice.* **A.** The relative mRNA levels of *Ces* are depicted for thioglycollate-elicited mouse macrophages, treated in culture for 4h with saline (vehicle, 0.1% v/v, white bars) or 100 ng/ml LPS (black bars). Increased expression of *Il1β* and *Tnf* mRNA demonstrate efficacy of LPS administration (inset). Results depict the mean ± SEM for three wells per treatment. All values were mathematically adjusted relative to the most abundant value (*Ces1c*, vehicle) set at 100%, so all bars in this graph can be

compared. * $P < 0.05$, significant effect of LPS treatment by Student's *t*-test. **B.** Hepatic levels of *Ces* mRNA in mice fed for 16 weeks a high-fat diet relative to mice fed a low-fat control diet, which is represented by the hatched line, set at 100%. Expressed in this manner, comparisons cannot be made between CES members in this panel. Results depict the mean ± SEM for n=6 mice per dietary group, * $P < 0.05$; **, *P<0.01;* ***, *P<0.001* significant effect of high-fat feeding as determined by Student's *t*-test. ND, not detected. NM, not measured.

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FIGURE 1

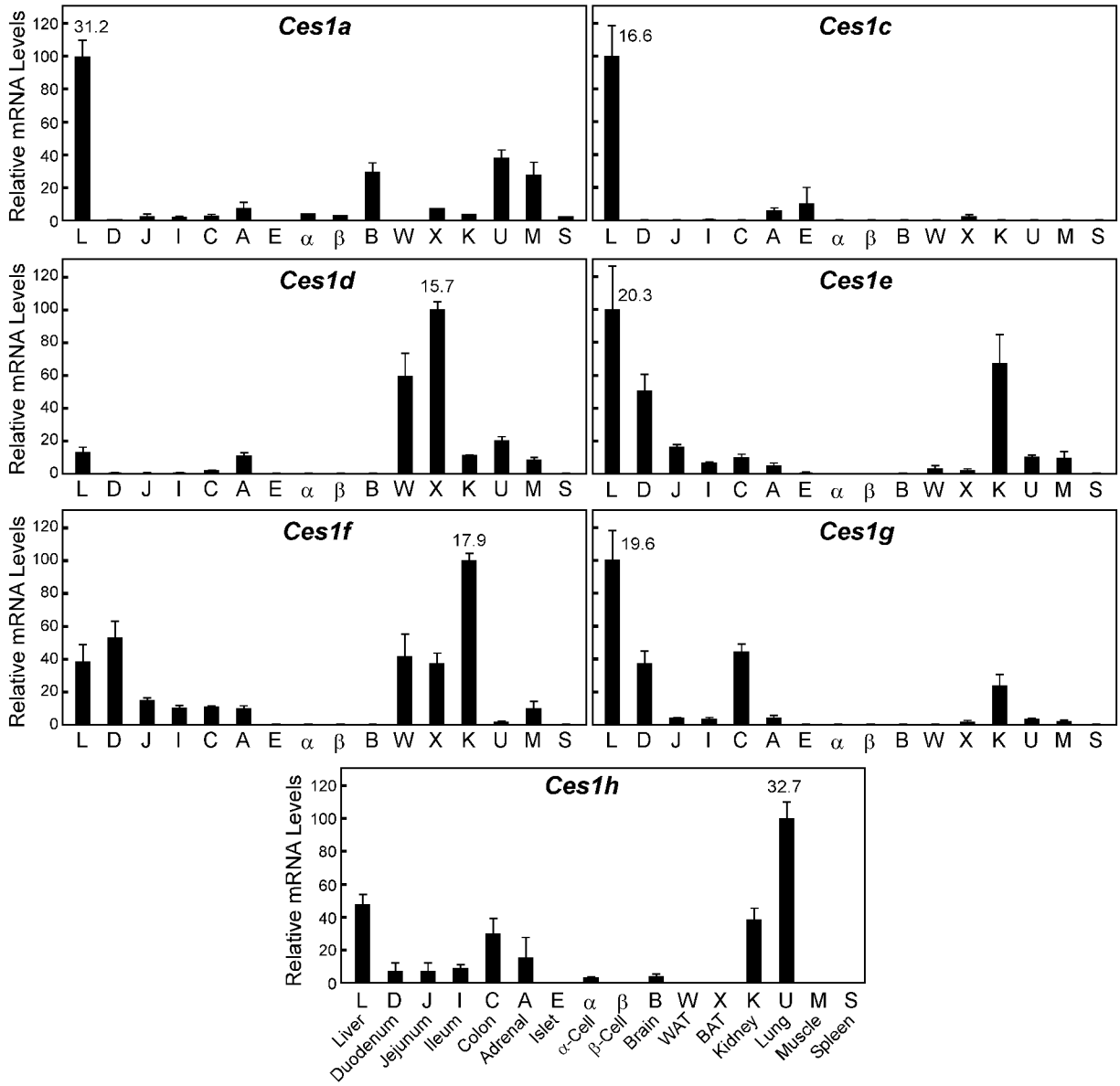

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FIGURE 2

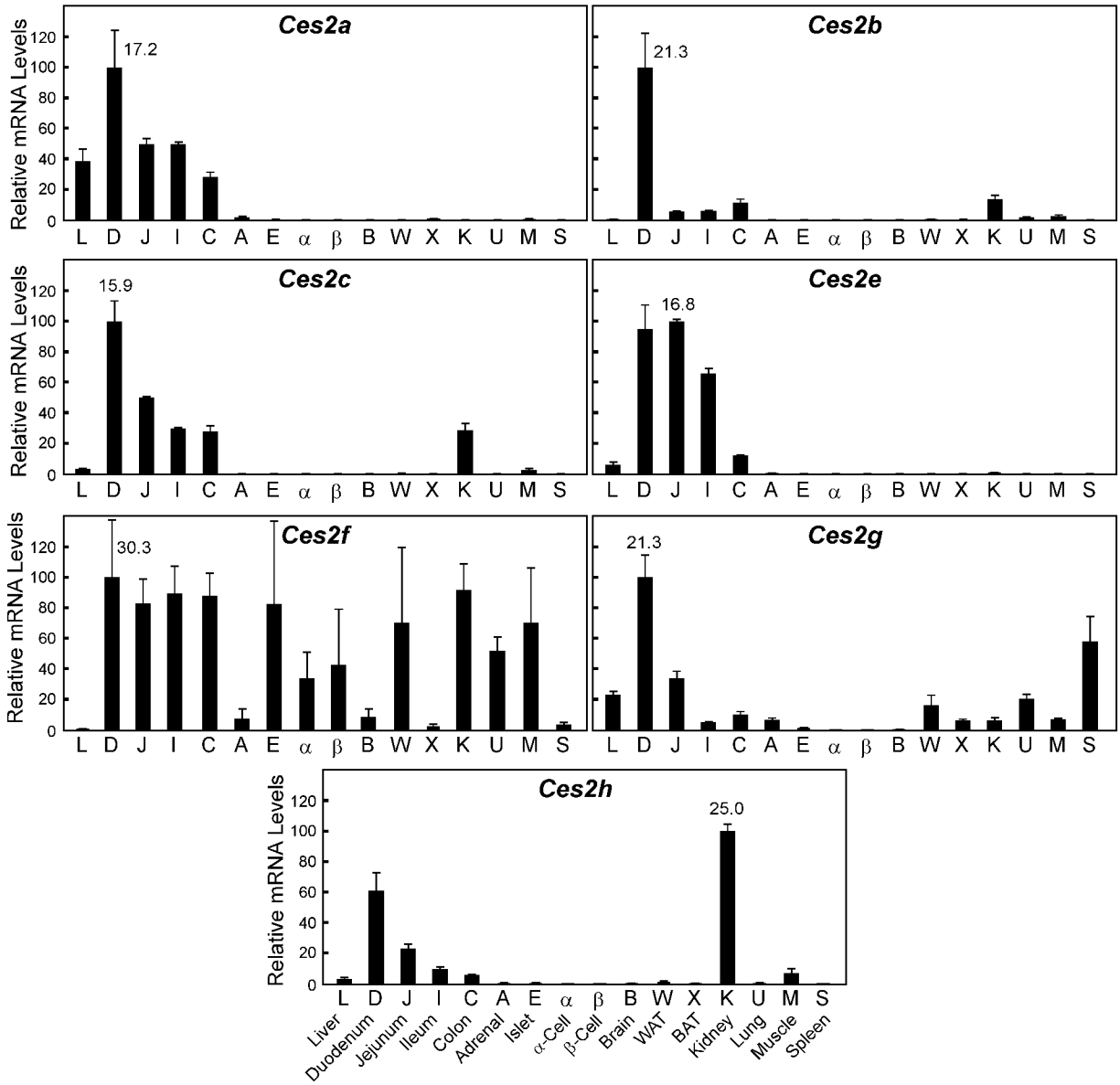

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FIGURE 3

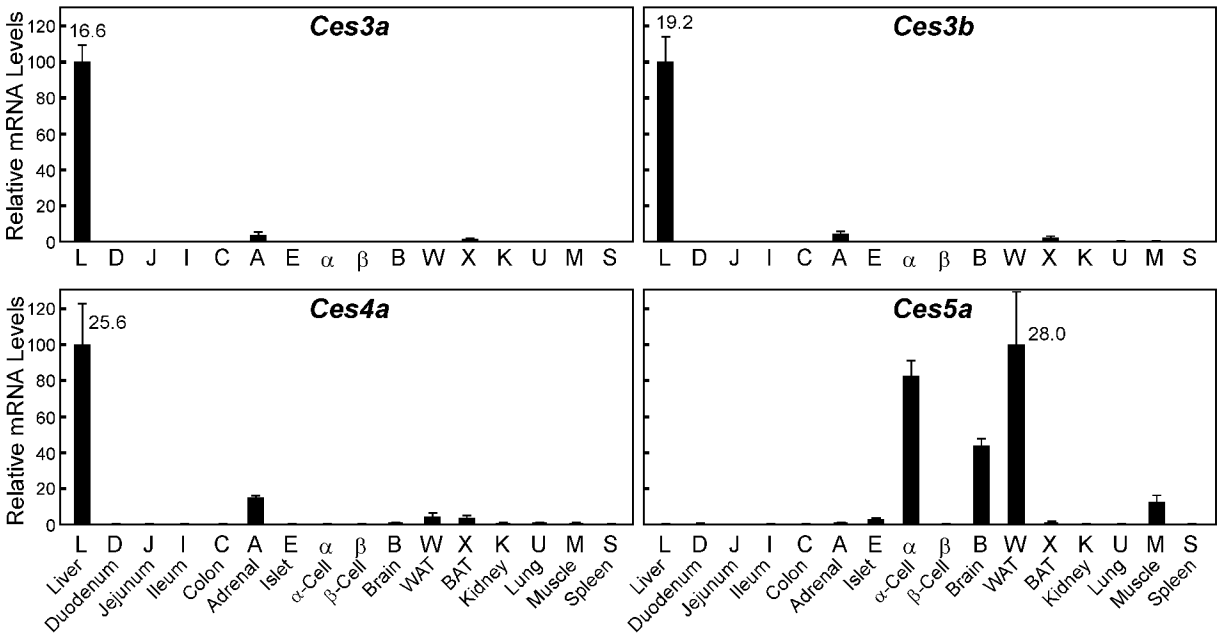

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FIGURE 4

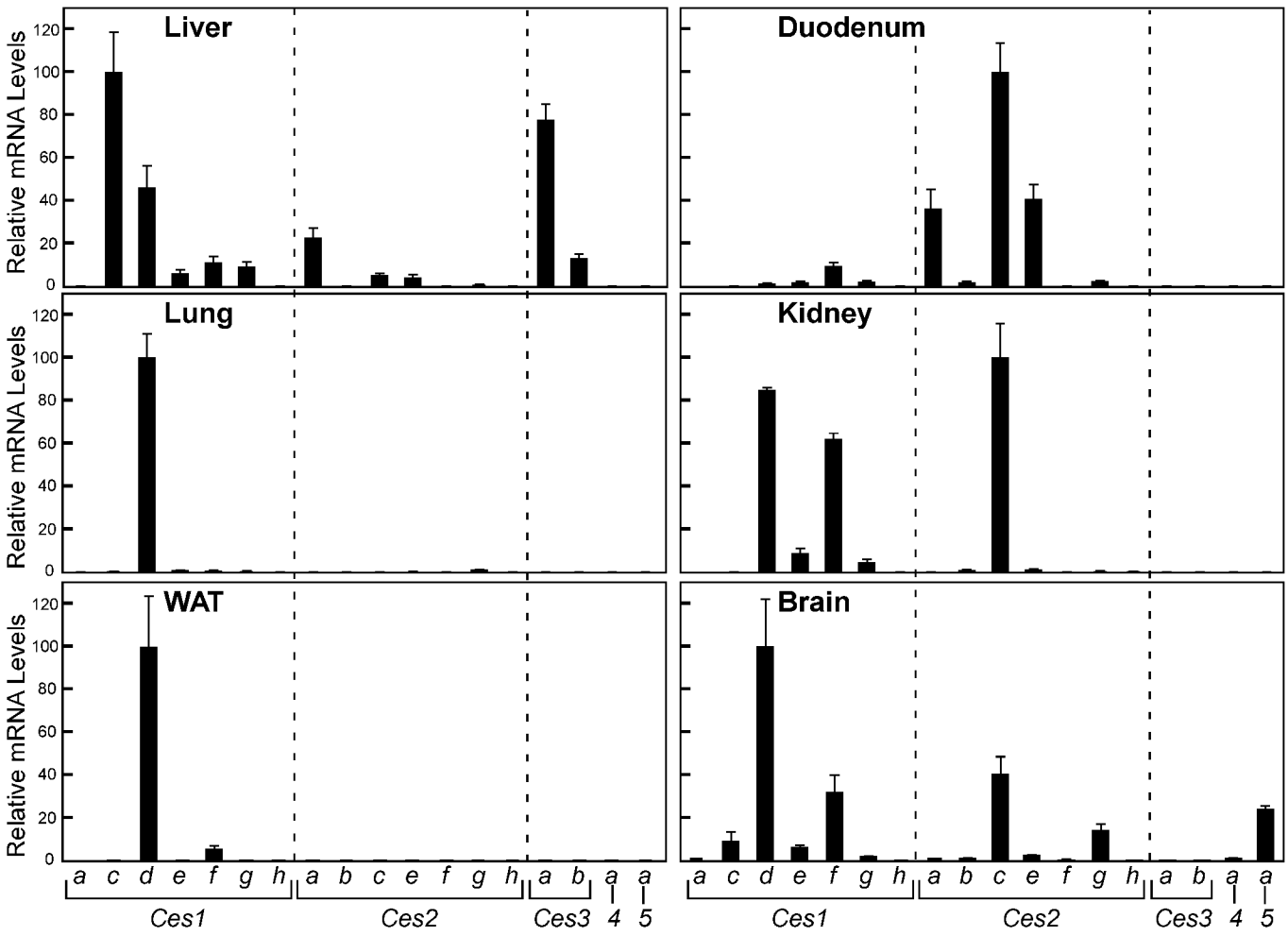

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FIGURE 5

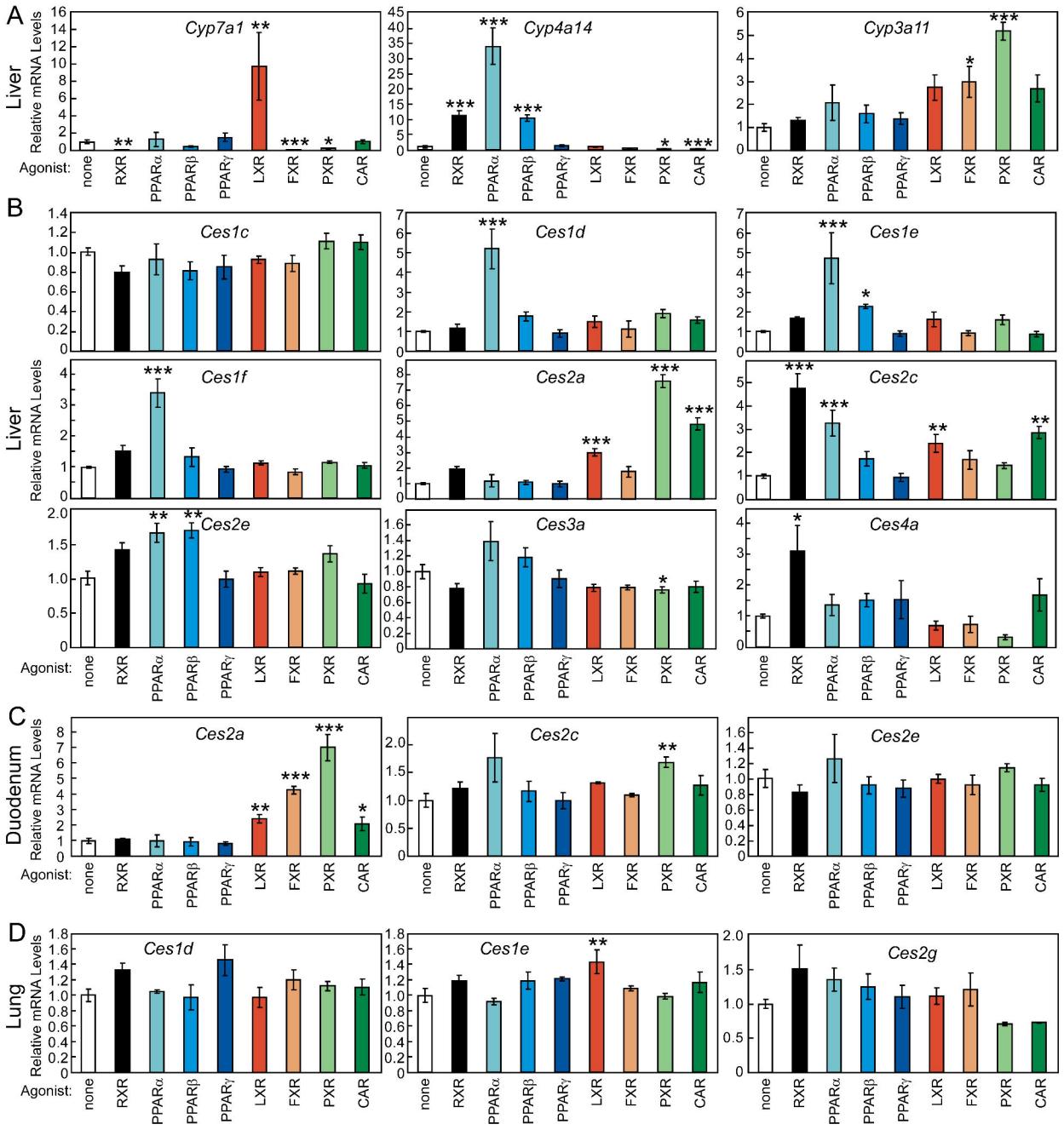

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FIGURE 6