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Cloning and Enzymatic Characterization of Human Carboxylesterases

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AFFIDAVIT

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Abstract

Carboxylesterases (CES) are enzymes of the serine hydrolase superfamily and are widely spread throughout the mammalian body. They are able to hydrolyse an ample array of substrates like esters, amides, and thioesters. In humans, 5 CES genes are known (CES1, CES2, CES3, CES4A, and CES5A). Over the last years, it has become more and more clear that CES family members have a dual role in xenobiotic as well as endogenous metabolism. However, most studies focused on CES1. The aim of this thesis was to screen human CES isoforms for their hydrolytic activities with endogenous as well as artificial lipid substrates. Furthermore, live cell imaging was performed to assess the localisation of CES4A isoforms in cells. The results show high activity of CES1- and CES2-containing cell lysates for all tested endogenous substrates. The experiments showed a strong dependency of CES1 and CES2 on the presence of Chaps, a bile acid-like detergents. Other isoforms only showed slight to no activity. Live cell imaging didn't show distinct localisation of CES4A isoform in the cell. However, reticular ER-like structures as well as early exosome-like clusters around the nucleus were observed. In conclusion, this study shows the broad substrate specificity of CES1 and CES2 for endogenous lipid esters as well as a possible role of bile acid in regulating CESs hydrolytic activities.

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1 Introduction

The mammalian metabolism is an incredibly complicated system with many different pathways. One of them is the lipid metabolism, which takes place in all cells but is most abundant in the liver, intestine and white adipose tissue. One part of this metabolism is the catabolism of lipids to release fatty acids, which can be used for energy production, membrane synthesis and much more. This is accomplished by specific enzymes capable of hydrolysing lipid esters. These enzymes are called hydrolases. The enzymes of the carboxylesterase family are hydrolases known to be able to catalyse these reactions. In mice, carboxylesterases are thought to play a role in the metabolism of xenobiotics but also of endogenous lipid substrates. In contrast, human carboxylesterases are thought to play a role mainly in the metabolism of xenobiotics. However, recent studies suggest an involvement of human carboxylesterases in endogenous lipid metabolism.

1.1 Mammalian Metabolism

Living organisms rely on a constant influx of energy for their basic functions such as muscle contraction, active transport of molecules and synthesis of macromolecules. In mammals, the majority of energy is taken in through food, broken down into absorbable components within the intestine and delivered to the bloodstream by the gut. Body cells are able to absorb energy sources and building blocks from the circulation and use them for their metabolism. Metabolism is the mechanism processing one substance into a different one, using or releasing energy. This process differentiates between two process directions: Anabolism, which uses energy to build structures, and catabolism, which generates useable energy by breaking down molecules. The three main macromolecules, which are used in this process, are proteins, carbohydrates, and lipids. (1)

1.2 Lipid-Metabolism

Lipids are a vital part of all living organisms and so is the lipid metabolism. This pathway regulates the use of lipids as a fuel source to maintain constant circulating glucose levels as well as the synthesis of building blocks for membranes, hormones and others. Dysfunctions in the regulation of these processes can lead to serious diseases such as the metabolic syndrome. In humans, de novo lipid synthesis plays a rather limited role as most lipids are ingested with our diet. Lipids are absorbed in the gut. Prior to their uptake, some lipids require their hydrolysis in the lumen of the intestine. Lipids and break down products are then absorbed into the mucosa cells. The enterocytes pack those lipids into lipoproteins, named chylomicrons, and release them into the bloodstream. Using receptors, the peripheral tissue connects to the apoproteins on the surface of those lipoproteins and extracts necessary fatty acids. Largely depleted remnant

chylomicrons then reach the liver and are absorbed. The liver itself acts as a central hub for lipid uptake, synthesis, turnover and release. Hepatocytes take up remnant chylomicrons, repack the lipids, and distribute very low-density lipoprotein vesicles again for peripheral tissues. (1) Excess lipids are stored as triacylglycerol (TAG) in white adipose tissue (WAT), which is comprised of adipocytes that contain massive lipid droplets (LD). From adipose tissues, lipids can get mobilized through lipolysis under starvation or nutritional undersupply. (2) Most lipids are transported and stored in ester form such as TAG, cholesteryl ester (CE) and retinyl ester (RE).

1.3 Triacylglycerol

TAGs belong to the class of neutral lipids (together with diacylglycerols, monoacylglycerols, REs and CEs) and are composed of the trivalent alcohol glycerol and 3 fatty acids (FAs) connected by ester bonds. In humans, FAs usually contain an even number of C-atoms. By far the most common FAs are fully saturated FAs, like palmitic acid (C16:0, number of C-atoms:number of double bonds) and stearic acid (C18:0). Within the unsaturated FAs, oleic acid (C18:1) and linoleic acid (18:2) are abundant. Humans are only able to synthesise monounsaturated FAs (with exception of arachidonic acid). Higher degrees of unsaturated FAs must be taken in with the diet and are called essential FAs. TAGs are very important for energy metabolism. One of their main functions is to store FAs in large LDs within the WAT. These FAs can then be used for ATP production, membrane synthesis and signalling molecules. To release FAs from TAGs, enzymes that hydrolyse the ester bonds between the FAs and the glycerine backbone are needed. This process is called lipolysis (3).

1.4 Cholesteryl ester

CEs are important membrane lipids. They represent the transport form for cholesterol. They consist of cholesterol with an FA esterified to its 3' hydroxyl group. As a membrane lipid, it is significant for the fluidity of the membrane. Cholesterol by itself is required for a diverse array of functions as it is a critical precursor for steroid hormones, vitamin D and bile acid. Cholesterol is mainly taken in through the diet. However, it is also synthesised de novo within the cells, which is a very energy consuming process. Cholesterol can't be degraded by most cells. It can only be eliminated by excretion via bile acids. CEs are transported via lipoproteins of any type but are most abundant within low-density lipoproteins (LDLs). Dysfunction of cholesterol transport, such as familial hypercholesterolemia with defect LDL-receptors, can lead to severe cholesterol disposition in the intima of the arterial wall, which eventually leads to pathological consequences such as atherosclerosis (3).

1.5 Retinyl ester

REs are the storage form of retinol. Retinol is per definition termed vitamin A. It plays a significant role in the mammalian visual-cycle and various signalling pathways and most importantly in the regulation of body growth. Vitamin A is an alcohol consisting of four isoprene units. For mammals including humans it is an essential component of nutrients. It is consumed as retinol, RE or as its precursor β-carotene. After absorption of β-carotene via the enterocytes, the precursor is cleaved into retinal, converted to retinol and esterified to RE. Vitamin A and RE are transported to the liver through chylomicrons. Within hepatocytes, retinol is esterified mostly with palmitate to retinylpalmitate (RP) for storage. The liver accumulates vitamin A in the ester form in large quantities. If needed, retinol can be mobilized by cleavage of the FA by a hydrolase and transported by its specific transport protein retinol-binding protein 4 (3).

1.6 Lipolysis

Lipolysis is the process in which TAG is catabolised. Within LDs, stored TAGs are hydrolytically cleaved to glycerol and FAs, which in turn can be used for multiple purposes (see above). This process is essential for energy supply as it provides FA for ATP production, which takes place in practically all tissues. However, TAG is most abundant in the white and brown adipose tissue, for energy storage and thermogenesis respectively. The enzymes involved in the hydrolysis of TAGs are called lipases. For complete hydrolysis of a TAG molecule three enzymes are required which form a catalytic cascade. The first and rate-limiting enzyme is adipose triglyceride lipase (ATGL a.k.a. PNPLA2) with its activator, comparative gene identification-58 (CGI-58). It catalyses the degradation of TAGs into diacylglycerols (DAGs) and fatty acids (FAs). The second enzyme of this cascade is hormone sensitive lipase (HSL), which cleaves DAG into monoacylglycerol (MAG) and a FA. The last hydrolytic step is carried out by monoacylglyceride lipase (MGL), converting MAG into glycerol and a FA. Before ATGL was discovered, HSL was thought to be the rate-limiting enzyme for TAG catabolism, as it is also capable of the hydrolysis of TAG. Together, ATGL and HSL make up 90% of the total lipolytic capacity in murine adipose tissue. While ATGL and HSL play a major role in TAG catabolism, other enzymes located on LDs and at the ER contribute to this process, such as enzymes of the PNPLA- and CES-families. (2; 3)

1.7 Carboxylesterases

Carboxylesterases (CES, EC 3.1.1.1) constitute a well-conserved enzyme-family containing an α/β-hydrolase-fold domain (4). They are expressed in many mammalian tissues (5). The catalytic triad within the active site, responsible for this hydrolytic process, consists of 3 amino acids: serine, histidine and glutamine (or aspartate). Ester bonds are targeted at the carbonyl carbon via the nucleophilic serine residue (6). They catalyse the hydrolysis of an ample array of endogenous and exogenous substrates containing ester, amide, and thioester bonds (7; 8). In particular the hydrolysis of esters plays an important role in mammals by cleaving the ester into the corresponding carboxylic acid and an alcohol. This generally results in more polar molecules thereby increasing their water solubility and promoting renal elimination (9). This is especially important for the metabolism and extraction of xenobiotics. CESs in mammals can be classified into 5 groups (CES1-5), based on their amino acid homology (**Table 1**). However, CES1 and CES2 family members are most commonly identified (8; 10). Furthermore, while significance of CES for the turnover and elimination of exogenously ingested esters, such as drugs and pesticides, is firmly established, its role in the endogenous lipid metabolism of mice was only recently demonstrated. Much less is known about endogenous substrates for human CESs as compared to their murine homologs (**Table 2**). Though mice CES isoforms show enzyme activity for TAGs, MAGs, REs and CEs, only for hCES 1 endogenous substrate activity was reported (TAG and CE hydrolysis). Most substrates known for human CES isoforms are xenobiotics like heroin, cocaine and sarin (11). As mentioned above, CESs are found in most mammalian tissues, including but not limited to liver, small intestine, lung, kidney, adipose tissue, testis, and macrophages. They are usually located within the endoplasmic reticulum or the cytoplasm (5).

Table 1: Human CES genes and their transcripts.

	Gene name	*Synonym*	*Transcript variant*	*Suggested name*	*NCBI RefSeq. Nucleotide*	*mRNA length*
1	CES1	ACAT, CE-1, CEH, CES2, hCE-1, HMSE. HMSE1, PCE-1, REH, SES1, TGH	CES1 isoform 1	CES1A1	NM_001025195	2,027
2			CES1 isoform 2	CES1A2	NM_001025194	2,024
3			CES1 isoform 3	CES1A3	NM_001266	2,021
4	CES2	CE-2, iCE, PCE-2	CES2 isoform 1	CES2A1	NM_003869	4,177
5			CES2 isoform 2	CES2A2	NM_198061	3,907
6	CES3	ES31 CES6, CES8	CES3 isoform 1	CES3A1	NM_024922	3,912
7			CES3 isoform 2	CES3A2	NM_001185177	3,903
8			CES3 isoform 3	CES3A3	NM_001185176	3,196
9	CES4A	CES6, CES8 CAUXIN, CES5, CES7, HEL126	CES4A isoform 1	CES4A1	NM_173815	2,296
10			CES4A isoform 2	CES4A3	NM_001190201	2,171
11			CES4A isoform 3	CES4A4	NM_001190202	1,981
12	CES5A	CAUXIN, CES5, CES7, HEL126	CES5A isoform 1	CES5A1	NM_001143685	2,285
13			CES5A isoform 2	CES5A2	NM_145024	2,135
14			CES5A isoform 3	CES5A3	NM_001190158	2,258

Table 2: Substrates and function identified for various mammalian CES isoforms, modified according to (11).

Mammal	*CES (Ces) gene*	*Current gene symbol(s)*	*Substrates and function (hydrolysis or detoxification)*
Human	CES 1	CES1, hCE-1, CES1A1, HU1	Heroin, cocaine[1], methyl phenidate[2], temocapril[3], CPT-11[4], flurbiprofen[5]
		CES1	Fatty acid ethyl ester synthase[6], sarin[7], ciclesonide[8], cholesteryl ester hydrolase[9], triacylglycerolhydrolase[9]
	CES2	CES2, hCE-2, HU2	Procaine[3], heroin, cocaine[1], temacapril[3] , CPT-11,6 flurbiprofen[5], doxazolidine[10]
	CES3	CES3	CPT-11[4]
Mouse	Ces1c	Es1, Ces-N	Lung surfactant convertase[11], CPT-11[12]
	Ces1d	Ces3	Triacylglycerol hydrolase[13]
	Ces1e	Es22, egasyn	β-glucuronidase binding in the liver endoplasmic reticulum[14], retinyl ester hydrolase[15]
	Ces1f	CesML1, TGH-2	Triacylglycerol hydrolase[16], monoacylglycerol[16] hydrolase[16], cholesteryl ester hydrolase[16], phospholipase[16]
	Ces1g	Ces1	Lipid metabolism[17]
	Ces2c	Ces2	Inducible liver acylcarnitine hydrolase[18]
Rat	Ces1c	Es1	Retinyl palmitate[19]
	Ces1d	Ces3	Cholesterol ester hydrolase[20], triacylglycerol hydrolase[16], retinyl ester hydrolase[21]
	Ces1e	ES-3	β-glucuronidase binding in the liver endoplasmic reticulum[22]
	Ces2a	Ces6	Intestinal first pass metabolism[23]
	Ces2c	Ces2	Inducible liver acylcarnitine hydrolase[18], intestinal first pass metabolism[23]
	Ces2e	Ces5	Intestinal first pass metabolism[23]
Cat	CES5A	CES7, cauxin	3-Methylbutanol-cysteinylglycine hydrolysis in urine releasing pheromone[24]
Rat, sheep	CES5A	CES7, cauxin	Lipid transfer reactions in epididymis[25]

[1] (33; 7; 29)
[2] (27)
[3] (25)
[4] (45; 23; 37; 39)
[5] (26; 24; 46)
[6] (53)
[7] (47)
[8] (38)
[9] (54)
[10] (55)
[11] (43; 31)
[12] (39)
[13] (52)
[14] (34)
[15] (28)
[16] (42)
[17] (50; 44)
[18] (49)
[19] (30)
[20] (48; 35)
[21] (36)
[22] (32)
[23] (41)
[24] (40)
[25] (51; 22)

1.8 Aim of the Thesis

It is firmly established that human CES isoforms cleave a wide variety of xenobiotics. More recent publications show hydrolytic activity of murine CES isoforms for endogenous lipid ester substrates. In mice, the important role of CES isoforms in lipid metabolism has been demonstrated. The aim of this thesis was to analyse several human CES isoforms for their activity on a variety of endogenous lipid ester substrates, with a focus on neutral lipid esters and to study the cellular location of human CES isoforms using live cell imaging.

2 Materials

Chemicals and Materials were mainly obtained from Merck (Darmstadt, Germany), Sigma Aldrich (St. Louis, MO), Roth GmbH (Karlsruhe, Germany) and New England Biolab (Ipswich, MA). Important solution compositions, cells, kits and plasmids are described below.

2.1 Buffers and Solutions

The following buffers and solutions were used for the lab work:

Name	Composition
10% SDS Separating Gel	12.3 mL ddH_2O, 7.5 mL 4x „lower buffer“, 9.9 mL 30 % Acrylamid, 300 µl 10% SDS, 27 µl N,N,N',N'-Tetramethylethylendiamin, 81 µl 10% APS
SDS Stacking Gel	5.9 mL ddH_2O, 2.5 mL 4x „upper buffer“, 1.5 mL 30% Acrylamid, 100 µl 10% SDS, 26 µl N,N,N',N'-Tetramethylethylendiamin, 80 µl 10% APS, 15 µl 0.5% „blue Dye“
4x SDS Sample Buffer	4 mL 100% Glycerin, 4 mL 20% SDS, 1 mL 2M Tris (pH 6.8), a spatula tip of bromphenol, 1 mL β-Mercaptoethanol and fill to 10 mL with ddH_2O
4x Lower Buffer	90.86 g Tris onto 500 mL with ddH_2O, pH 8.8
4x Upper Buffer	30.29 g Tris onto 500 mL with ddH_2O, pH 6.8
Caps Buffer	4.42 g Caps, 200 mL Methanol, fill to 2000 mL with ddH_2O
Tris-Glycin Running Buffer	50.4 g Tris, 241.7 g Glycin, 16.7 g SDS, fill to 2000 mL with ddH_2O
Ampicillin-Agar Media	10 g Tryptone, 5 g Yeast extract, 10 g NaCl, 1 mL 100 µg/µl Ampicillin, fill to 1 L with ddH_2O, pH 7.4
Ampicillin-Agar Plates Media	10 g Tryptone, 5 g Yeast extract, 10 g NaCl, 15 g Agar, 1 mL 100 µg/µl Ampicillin, fill to 100 mL with ddH_2O, pH 7.4
PBS Buffer	8 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, 0.27 g KH_2PO_4, fill to 1000 mL with ddH_2O
HSL Solution	85.57 g Sucrose, 154 mg DTT, 372 mg EDTA-NA_2, fill to 1000 mL with ddH_2O, pH 7

TBE Buffer	10.8 g Tris, 5.5 g Boric acid, 0.7 g EDTA-NA_2, fill to 1000 mL with ddH_2O, pH 8
TST Buffer	10 mL Tween 20, 87.6 g NaCl, 15.76 g Tris HCl, fill to 1000 mL with ddH_2O, pH 7.4
BTP Buffer	13.97 g K_2HPO_4, 2.69 g KH_2PO_4, fill to 1000 mL, pH 7.4
Coomassie Blue Solution	0.25 mL Coomassie Blue, 7.5 mL 80 % Acetic Acid, 50 mL Ethanol, fill to 100 mL with ddH_2O
Destain Solution	30 mL Ethanol, 10 mL Acetic Acid, fill to 100 mL with ddH_2O
SOC Medium	20 g Tryptone, 5 g Yeast Extract, 0.5 g NaCl, 186 mg KCl, 3.6 g Glucose, fill to fill to 1000 mL with ddH_2O

2.2 Cells

The following cells were used:

Name	**Company**
Cos7 ATCC® CRL-1651™	American Tissue Culture Collection, Manassas, WV

2.3 Kits

The following kits were used:

Name	**Company**
QIAamp DNA Mini Kit	QIAGEN GmbH, Hilden, Germany
NucleoBond Xtra Midi / Maxi	QIAGEN GmbH, Hilden, Germany
E.Z.N.A. Gel Extraction Kit	Omega Bio-tek Inc., Norcross, GA
HR Series NEFA-HR(2) Kit	Wako Life Sciences Inc. Richmond, VA
BIORAD Bradford Protein Assay Kit	Bio-Rad Laboratories, Inc., Hercules, CA
Clarity™ Western ECL Blotting Substrate	Bio-Rad Laboratories, Inc., Hercules, CA

2.4 Plasmids

The following plasmids were used:

Name	Company
pcDNA4/HisMaxC	Life Technologies Ltd, Paisley, Great Britain
pCI-Neo	From Prof. Richard Lehner, University of Alberta, Edmonton, Canada
pECFP-N1	BD Bioscience, San Jose, CA

2.5 Live Cell Imaging Marker

The following Markers were used:

Name	Company
HCS LipidTOX Deep Red Neutral Lipid Stain	Thermo Fisher Scientific, Waltham, MA
LysoTracker Deep Red	Thermo Fisher Scientific, Waltham, MA
pDsRed2-ER Vector	Clonetech, Mountain View, CA

2.6 Antibodies

The following antibodies were used:

Name	Company
Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody produced in mouse	Sigma Aldrich, St. Louis, MO
Anti n-term 6xHis AB, from mouse	GE Healthcare, Chicago, IL
ECL Mouse IgG, HRP-linked whole AB, from sheep	GE Healthcare, Chicago, IL

3 Methods

3.1 Polymerase Chain Reaction

For the amplification of targeted genes, polymerase chain reaction (PCR) was used.
The human CES4A1 as well as CES4A3 and CES4A4 coding sequences (CDS) were amplified using cDNA of human WAT and human liver, respectively. Amplification was done in a total volume of 25 µl containing 1 µl of cDNA, 5 µl 5x PCR buffer, 5 µl betaine solution (5M), 1µl of respective forward and reverse primers for pcDNA4/HisMaxC (10 nM, **Table 4**), 1 µl of dNTP's (10mM), 11.7 µl of ddH_2O and 0.3 µl of Phusion polymerase (Thermo Fisher Scientific, Waltham, MA). Nested PCR was performed using the temperature programmes listed in **Table 3**. PCR products were separated using agarose gel electrophoresis. Distinct DNA bands were cut out and DNA eluted via E.Z.N.A. Gel Extraction Kit (Omega Bio-tek Inc., Norcross, GA). Eluted CES amplicons were digested to screen for sequence specific restriction sites.
An additional PCR was performed for live cell imaging plasmids. CES4A isoforms containing pcDNA4/HisMaxC plasmids were used as template with primers for pECFP-N1 vector (10 nM, **Table 1**). An annealing temperature of 62 °C was used for all CES4A isoforms.

Table 3: Temperature programme of the nested PCR for CES4A isoform CDS amplification.

	hCES4A1	*hCES4A3*	*hCES4A4*
1. PCR			
Initial denaturation	95 °C, 10'	95 °C, 10'	95 °C, 10'
Denaturation	95 °C, 30"	95 °C, 30"	95 °C, 30"
Annealing	Gradient: 59 – 69 °C, 8 steps	Touch Down: 70 – 60 °C, 10 cycles 60 °C, 25 cycles	Gradient: 59 – 70 °C, 8 steps
Extension	72 °C, 1'	72 °C, 1'	72 °C, 1'
Final extension	72 °C, 10'	72 °C, 10'	72 °C, 10'
Cycles	35	35	35
2. PCR			
Initial denaturation	95 °C, 10'	95 °C, 10'	95 °C, 10'
Denaturation	95 °C, 30"	95 °C, 30"	95 °C, 30"
Annealing	66.5 °C, 30'	Touch Down: 72 – 60 °C, 12 cycles 60 °C, 23 cycles	64 °C, 30'
Extension	72 °C, 1'	72 °C, 1'	72 °C, 1'
Final extension	72 °C, 10'	72 °C, 10'	72 °C, 10'
Cycles	35	35	35

Table 4: CES4A isoform specific primer used for PCR.

Primer	*Target*	*Target Vector*	*Direction*	*Restriction Site*	*Sequence*
hCES4A1_NotI_fw	CES4A1	pcDNA4	Forward	NotI	GGCGGCCGCTCAGGTGGATTCTGTGCTGGAGC
hCES4A1-4_XbaI_rv	CES4A1, CES4A4	pcDNA4	Reverse	XbaI	GTCTAGATCATGCCCACTCTTGTGGTAAA
hCES4A3-4_NotI_fw	CES4A3, CES4A4	pcDNA4	Forward	NotI	GGCGGCCGCTCTACGTCAGCACGCGGGA
hCES4A3_XbaI_rv	CES4A3	pcDNA4	Reverse	XbaI	GTCTAGATTAGAATTCCGTCTGCTTCTCAG
hCES4A1_XhoI_fw	CES4A1	pECFP-N1	Forward	XhoI	GTGCTCGAGATGAGGTGGATTCTGTGCTGGA
CES4A1-4_AbeI_rv	CES4A1 CES4A4	pECFP-N1	Reverse	AbeI	CACACCGGTGCTGCCCACTCTTGTGGTAAAATCC
CES4A3-4_XhoI_fw	CES4A3 CES4A4	pECFP-N1	Forward	XhoI	GTGCTCGAGATGTACGTCAGCACGCGGGA
CES4A3_AbeI_rv	CES4A3	pECFP-N1	Reverse	AbeI	CACACCGGTGCGAATTCGGTCTGCTTCTCAGGT

3.2 Cloning

After PCR, CES CDS amplicons were ligated into the multiple cloning site of an expression vector maintaining the reading frame and transformed into *E.Coli*.
Amplicons and vectors were digested using their respective restriction enzymes (**Table 4**). To purify the DNA, a gel electrophoresis with subsequent DNA extraction via E.Z.N.A. Gel Extraction Kit was performed. Concentration of the extracted DNA was measured via NanoDrop ND-1000 (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The isolated inserts (amplicons) and vectors (pcDNA4/HisMaxC and pECFP-N1) were ligated in an 5:1 ratio (insert:vector molecules) in a total volume of 20 µl containing 2 µl 10x T4 ligation buffer, 1µl T4 ligase (Invitrogen, Carlsbad, CA) and the residual amount of ddH_2O. The ligation was incubated at room temperature (RT) for an hour.
For transformation, the chemical competent *E.Coli* DH5α (Thermo Fisher Scientific, Waltham, MA) cells were allowed to thaw on ice and incubated with 5 µl of the corresponding ligation mixture for half an hour on ice. Cells were then heat shocked in a water bath at 42 °C for 30

seconds and regenerated on 37 °C with 200 µl of pre-warmed SOC medium for one hour. To select positively transformed cells, LB-agar plates containing an antibiotic agent matching the vector specific antibiotic resistance (ampicillin, kanamycin) were used to plate 50 µl of the cell suspension.
For isolation of plasmid DNA, 5 mL of LB-media containing the respective antibiotic agent were inoculated with single colonies picked from the LB-agar plates and incubated overnight at 37 °C under constant shaking. The plasmids were isolated using the QIAamp DNA Mini Kit and restriction digested for insert control. Plasmids carrying the insert were sequenced using standard promoter site primers. For higher plasmid yield, positively transformed cells with correct ligated plasmids were cultivated in 300 mL LB-media (37 °C, under constant shaking, containing respective antibiotics) and isolated using the NucleoBond® Xtra Midi/Maxi Kit.

3.3 Mammalian Cell Line Handling

Cos7 cell line were cultivated, maintained and harvested for further use. All sterility-demanding methods were done under a laminar flow. To cultivate Cos7 cells, a nitrogen culture tube was thawed at 37 °C and transferred into 7 mL of pre-warmed (37 °C) medium (Dulbecco's Modified Eagle Medium, DMEM, Life Technologies, Carlsbad, CA) containing 10 % fetal bovine serum (FBS) and 1% PenStrep (10,000 U/mL) (+/+, medium +FBS +antibiotics). The cell suspension was centrifuged at 1200 rpm and the pellet re-suspended in 2 mL of +/+ medium. Cell suspension was transferred into a 175cm² cell culture flask filled with 15 mL of prewarmed +/+ medium. Cells were incubated at standard conditions (37 °C, 7% CO_2, and 95% relative humidity). After growth to about 95% confluency, cells were split. To achieve this, cells were gently washed with phosphate-buffered saline solution (PBS). Then, cells were detached from the bottom of the flask by incubation with 3 mL of trypsin (Life Technologies, Carlsbad, CA) solution for 3 minutes. Then, 7 mL of +/+ medium were added and after gentle tapping of the flask, the suspension was transferred into a 50 mL tube. After centrifugation at 1200 rpm, the pellet was re-suspended in 10 mL +/+ medium and split in a ratio of 1:5 into new flasks containing +/+ medium.

3.4 Protein Expression

The following method was used to express recombinant proteins by transfection of Cos7 cells. Cos7 cells were plated in cell culture dishes (10 cm diameter) with $9x10^5$ cells per dish, containing 5 mL +/+ DMEM and cultivated overnight. For transfection, 6 µg of plasmid DNA in 300 µl of -/- medium were transferred into a solution containing 27 µl Metafectene (Biontex Laboratories GmbH, Munic, Germany) in 300 µl -/- medium and incubated at RT for 20 minutes. In the meantime, the medium of the cell culture dishes was replaced with 5 mL of +/- medium. After incubation, the transfection solution was added homogenously to the

incubation medium and incubated at 37 °C. To minimize the toxic effects of Metafecten, the medium was replaced by 5 mL of +/+ DMEM after 4 hours. After 48-72 h, cells usually showed maximum expression. At this point, cells were used for live cell imaging or lysed. For the latter, cells were washed with PBS and then suspended in 1 mL PBS using a cell scraper. The cell suspension was then centrifuged at 1,200 rpm, the pellet re-suspended in 100 µl HSL-solution and lysed on ice using an ultra sonicator (Sonicator S-4000, QSonica). Lysates were treated 3 times in 10-second-intervals at amplitude 1. After that, cell suspensions were centrifuged at 1,000 rpm for 10 minutes and the pellet containing the nuclei was discarded. Determination of protein concentration was done using the BIORAD Bradford Protein Assay kit. Cell lysate were stored at -20 °C until further use.

3.5 Live Cell Imaging

For localisation experiments, 6-well cell culture plates were used. A sterile microscope cover slip was placed in the well for transfer of cells to the microscope dish. Cos7 cells were cultivated ($1.5x10^5$ cells in 1 +/+ medium) for 24 hours and then transfected with fluorescent-labelled protein-encoding plasmids (1 µg plasmid in 50 µl -/- medium mixed with 4.5 µl Metafecten in 50 µl -/- medium) as described in the previous chapter (Protein Expression). For endoplasmic reticulum (ER) tracking, pDsRed2-ER-vector (Clonetech, Mountain View, CA) was co-transfected (0.5 µg target plasmid + 0.5 µg ER tracker plasmid). After 48h, cells were incubated with 1 mL +/+ medium containing LysoTracker (1:20,000, Thermo Fisher Scientific, Waltham, MA) or HSC LipidTOX (1:2,000, Thermo Fisher Scientific, Waltham, MA) for 30 min for lysosome and LD tracking, respectively. After washing two times with 1 mL of +/+ medium, the cover slips were removed and analysed using a laser scanning spectral confocal microscope (Leica TCS SP2).

3.6 Western Blotting Analysis

For the analysis of protein expression, Western blotting analysis was used. 20 µg of protein lysates were mixed with 5 µl of 4x SDS sample buffer and filled to 20 µl using HSL-buffer before denaturation at 95 °C for 5 minutes. After that, proteins were separated together with a protein standard (Precision Plus Protein™ All Blue Prestained Protein Standards, Bio-Rad Laboratories, Inc., Hercules, CA) by gel electrophoresis using a 10 % SDS-polyacrylamide gel. Protein separation was performed at 25 mA for one hour, submerged in Tris-Glycine running buffer. The separated proteins were blotted at 200 mA onto a Polyvinylidene fluoride membrane (submerged in CAPS-buffer). To prevent unspecific antibody (AB) binding, the membrane was blocked with a 10 % milk powder solution for one hour at RT or overnight at 4 °C. The membrane was then incubated for one hour with the respective AB at RT (**Table 5**). After that,

the blot was washed 3 times with TST-solution for 10 minutes each. The membrane was developed with Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, Inc., Hercules, CA) and detected with the ChemiDoc Touch Imaging System or x-ray films. The membrane was stained with Coomassie Brilliant Blue for loading control.

Table 5: Antibodies used for Western Blot

Name	*Target Epitope*	*Donor Organism*	*Horseradish Peroxidase coupled*	*Dilution*
ANTI-FLAG	Flag	Rabbit	Yes	1:5000
Anti n-term His	n-term His	Mouse	No	1:5000
ECL Mouse IgG	IgG mouse	Rabbit	Yes	1:10000

3.7 Enzyme Assays

The following assays were performed to determine the hydrolytic activity of cell lysates containing different expressed CES isoforms. LacZ (β-galactosidase, EC 3.2.1.23) containing lysates were used to determine endogenous hydrolytic activity of protein overexpressing cells. HSL-buffer was used as blank for background correction.

3.7.1 p-Nitrophenylacetat-Assay

To determine hydrolytic activity for the artificial substrate p-nitrophenylacetat (pNPA), 3 mM of pNPA was prepared in BTP-buffer in the absence or presence of 60 mM Chaps. Then, 50 µl of lysate was incubated with 50 µl of substrate for 5 min at 37 °C in a 96 well plate. Absorbance at 405 nm was measured immediately after incubation. Using the Lambert and Beers equation ($E=\varepsilon \cdot c \cdot I$) the amount of cleaved substrate was calculated using ε_{405} = 12,000 $M^{-1}cm^{-1}$ and I = 6 mm.

3.7.2 Monoacylglyceryol Hydrolase Assay

For the MAG hydrolase activity assay, the substrate (1 mM 1(3)-oleoyl-rac-glycerol, racOG) was prepared in BTP-buffer in the absence or presence of 16 mM Chaps. Substrates were sonicated (2x for 10 seconds at amplitude 1, Sonicator S-4000, QSonica), and 2 % Bovine Serum Albumin (BSA) (FA free) was added. Then, 50 µl of substrate were incubated with 50 µl of lysate (1 mg protein/mL) for 30 min at 37°C. To determine the amount of cleaved FA, the HR Series NEFA-HR(2) Kit together with a standard curve and linear regression analysis was applied. Absorbance was measured at 562 nm.

3.7.3 Cholesteryl Ester Hydrolase Assay

To determine CE hydrolase (CEH) activity, a substrate containing 208 µM cholesteryl oleate (CO) with 0.2 µCi of ^{3}H labelled CO, 45 mM phosphatidylcholine:phosphatidylinositol (PC:PI, 3:1) and 16 mM Chaps was used. The substrate was prepared in BTP-buffer, sonicated (Virsonic 475, Virtis) two times for 10 seconds at amplitude 1 and 2 % BSA (FA free) were added. Then, 100 µl substrate were mixed with 100 µl of lysate (0.4 mg protein/mL) and incubated in a water bath under gentle agitation at 37 °C for one hour. The reaction was terminated by adding 3.25 mL of methanol:chloroform:n-heptane solution (10:9:7) and 1 mL of 0.1 M K_2CO_3 buffer (pH 10.5, with boric acid). Samples were vigorously vortexed two times for 5 seconds and centrifuged at 2,500 rpm for 10 minutes. To analyse the amount of free cholesterol, 200 µl of the upper phase were pipetted into a scintillation vial containing 2 mL of scintillation cocktail. In addition, a vial with 20 µl of substrate instead of sample phase was also prepared. Radioactivity was measured using a scintillation counter (Tri-Carb 2100TR, Packard Instrument Company, Downers Grove, IL). Hydrolytic activities were calculated by utilising the formula below.

$$\frac{(cpm\ sample - cpm\ blank) * (V1 \div V2)}{(cpm\ substrate\ \div\ nmol\ FA) * mg\ protein * 0.715 * t} = \frac{nmol\ FA}{mg * h}$$

Cpm	=	counts per minute
V1/V2	=	volume of upper phase / measured volume (2.45 mL / 0.2 mL = 12.25)
nmol FA	=	nmol of FA cleavable of the substrate
mg protein	=	amount of protein in used lysate [mg]
0.715	=	extraction correction factor
t	=	incubation time [h]

3.7.4 Retinyl Ester Hydrolase Assay

To determine RE hydrolase (REH) activity, three different substrates were used. The first consisted of 600 µM RP and 80 mM Chaps. The second contained 600 µM RP with 600 µM PC. The third consisted of 600 µM RP, 90 µM PC and 16 mM Chaps. All substrates were prepared and sonicated (2x for 10 seconds at amplitude 1, Sonicator S-4000, QSonica) in BTP-buffer at a pH of 7.5 and contained 2 % FA free BSA (added after sonication). 100 µl of substrate were mixed with 100 µl of lysate (1 mg protein/mL) and incubated for one hour at 37 °C under gentle shaking. After incubation, the reaction was terminated by adding 100 µl of methanol containing 1.5 µM of internal standard (retinyl acetate). For extraction, 1 mL of hexane was

added, the mixture shaken for 10 min upside down and centrifuged for 5 min at 5000 rpm. 800 µl of the organic phase were evaporated using a SpeedVac and dissolved in 500 µl of chloroform:methanol (2:1) containing butylhydroxytoluene. 20 µl were injected into a HPLC system consisting of a Waters e2695 Separation Module, a column oven (at 25°C) with a reversed phase column (YMC-Pro C18 150x4.6mm, S-5µm, 12nm; YMC Europe GmbH, Dinslaken, Germany) and a Waters 2475 Fluorescence Detector (Waters Corporation, Milford, MA). By utilising the internal standard, the amount of retinol was calculated.

3.7.5 Triacylglycerol Hydrolase Assay

To analyse TAG hydrolase (TGH) activity, 0.3 mM triolein containing 0.5 µCi ^{3}H labelled triolein and 45mM PC:PI was used as substrate. In some cases, 16 mM Triton-X-100 or 16 mM Chaps was added. BTP-buffer was used for all preparations. After sonication (2x for 10 seconds at amplitude 1, Virsonic 475, Virtis) 2% BSA (fatty acid free) were added. 100 µl substrate and 100 µl lysate (1 mg protein/mL) were mixed and incubated at 37 °C for one hour under gentle shaking using a water bath. The reaction was terminated by adding 3.25 mL of methanol:chloroform:n-heptane solution (10:9:7) and 1 mL of 0.1 M K_2CO_3 buffer (pH 10.5, with boric acid). Samples were vigorously vortexed two times for 5 seconds and centrifuged at 2,500 rpm for 10 minutes. To analyse the amount of free FAs, 200 µl of the upper phase were pipetted into scintillation vials containing 2 mL of scintillation cocktail. In addition, a vial with 20 µl of substrate instead of sample was also prepared. Radioactivity was measured using a scintillation counter (Tri-Carb 2100TR, Packard Instrument Company, Downers Grove, IL) and calculated using the formula depicted above.

3.7.6 Hit Finder

To assess hydrolytic activities for MAG and DAG as well as phospholipids including diphosphatidylglycerol, the following substrates were used: phosphatidylcholine, phosphatidic acid, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, cardiolipin, n-acyl-phosphatidylethanolamine, bis(monoacylglycerol)phosphate and bis(diacylglycerol)phosphate. Two mM of substrates were prepared in BTP-buffer containing 16 mM Chaps, sonicated (2x for 10 seconds at amplitude 1, Virsonic 475, Virtis) and 1 % BSA (FA free) was added. Then, 100 µl of substrate were incubated with 100 µl lysate (1 mg protein/mL) for one hour at 37 °C. Hydrolytic activity was determined by measuring the free FA content using the HR Series NEFA-HR(2) Kit. For calculation a standard curve was prepared and the amount determined by linear regression.

3.8 Statistical Methods

Data are expressed as mean with standard deviation. Statistical significance was determined by Student's two-tailed t-test. Difference was considered statistically significant at the following p-values: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

4 Results

To investigate the role of human CES isoforms in lipid hydrolysis, the needed CDS were cloned into expression vectors.

4.1 Cloning of Human CES4 Isoforms

First, nested PCR was performed for CDS amplification of human CES4A isoforms. Human WAT cDNA and human liver cDNA were used as templates for CES4A1 as well as CES4A3 and CES4A4 amplification, respectively. After amplification, amplicons were separated by size on a 1.5 % agarose gel. **Figure 1** shows the separated bands of the CES4A isoforms at their expected sizes of 1406 bp, 1391 bp and 1124 bp for CES4A1, CES4A3, and CES4A4, respectively. Bands were cut out, extracted and control digested. All amplicons showed their expected restriction fragment (**Figure 2**) and undigested amplicons were used for ligation procedure. Vectors and inserts (amplicons) were digested for ligation. After separation and extraction, the inserts were ligated into the corresponding vector, transformed into *E.Coli*, incubated and isolated. **Figure 3** and **Figure 4** show the final plasmids after control digest. Both plasmids showed their expected fragments. Amplification of CES5A isoforms was not accomplished and therefore not further pursued for this thesis.

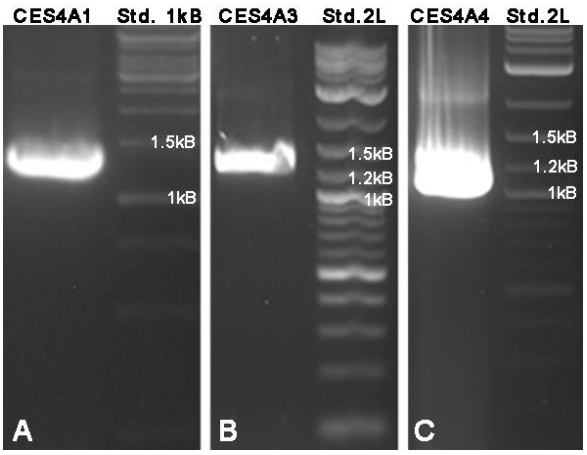

Figure 1: CES4A isoform amplicons after nested PCR. Nested PCR was performed with human WAT cDNA and human liver cDNA. After amplification, amplicons were separated on a 1.5 % agarose gel together with DNA standard ladder (Std. 1kb = GeneRuler 1 kb DNA Ladder, Fermentas, Burlington, Canada; Std. 2Log = 2-Log DNA Ladder (0.1-10.0 kb), New England BioLabs, Ipswich, Ma). CES4A isoform amplicons were found at their expected sizes. (A) CES4A1 = 1406 bp. (B) CES4A3 = 1391 bp. (C) CES4A4 = 1124 bp.

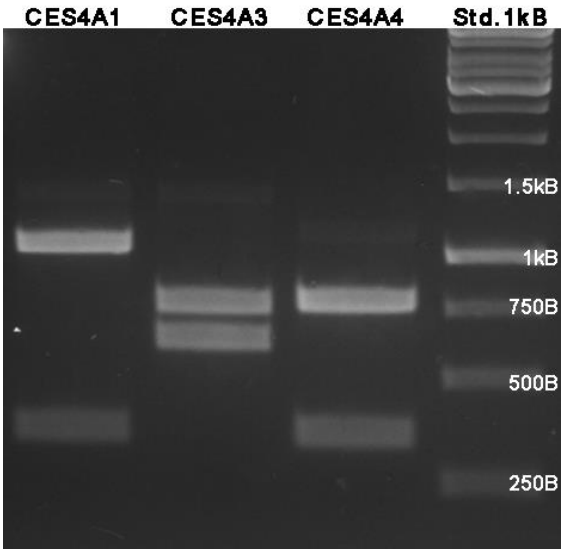

Figure 2: Control digest of CES4A isoforms. PCR amplicons were eluted using the E.Z.N.A. Gel Extraction Kit (Omega Bio-tek Inc., Norcross, GA) and then digested with EcoRI restriction enzyme (New England Biolab (Ipswich, MA). After incubation for 1 h at 37 °C the fragments were separated on a 1.5 % agarose gel together with DNA standard ladder (Std. 1kb = GeneRuler 1 kb DNA Ladder, Fermentas, Burlington, Canada. CES4A isoform fragments were found at their expected sizes. CES4A1 = 1057 + 350 bp. CES4A3 = 763 + 629 bp. CES4A4 = 775 + 350 bp.

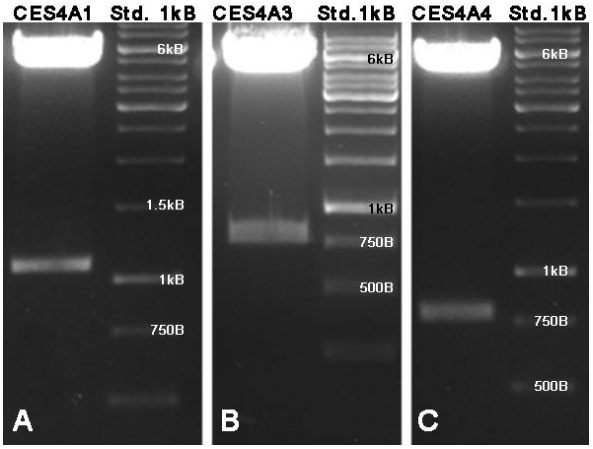

Figure 3: Control digest of CES4A isoforms pcDNA4/HisMaxC vectors. PCR amplicons were eluted using the E.Z.N.A. Gel Extraction Kit (Omega Bio-tek Inc., Norcross, GA), amplicons and pcDNA4/HisMaxC vector digested with NotI and XbaI, and ligated using T4 ligase (Sigma Aldrich, St. Louis, MO). After transformation of the plasmids into Cos7 cells and incubation, the plasmids were isolated with the DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) and then control digested with NotI and XbaI (CES4A3), and NotI and EcoRI (CES4A1, CES4A4). Constructs were separated on a 1.5 % agarose gel together with DNA standard ladder (Std. 1kb = GeneRuler 1 kb DNA Ladder, Fermentas, Burlington, Canada). CES4A insert fragments were found at their expected sizes. (A) CES4A1 = 1063 bp. (B) CES4A3 = 769 bp. (C) CES4A4 = 781 bp.

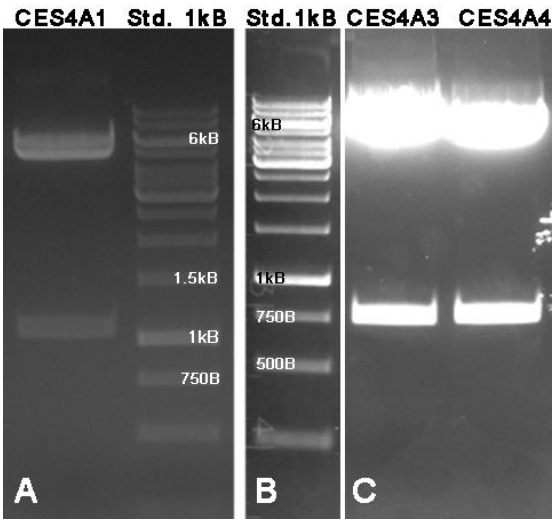

Figure 4: Control digest of CES4A isoforms pECFP-N1 vectors. PCR amplicons were eluted using the E.Z.N.A. Gel Extraction Kit (Omega Bio-tek Inc., Norcross, GA), amplicons and pECFP-N1 vector digested with XhoI and AbeI, and ligated using T4 ligase (Sigma Aldrich, St. Louis, MO). After transformation of the plasmids into Cos7 cells and incubation, the plasmids were isolated with the DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) and then control digested with EcoRI. Constructs were separated on a 1.5 % agarose gel together with DNA standard ladder (Std. 1kb = GeneRuler 1 kb DNA Ladder, Fermentas, Burlington, Canada). CES4A insert fragments were found at their expected sizes. (A) CES4A1 = 1062 bp. (C) CES4A3 = 769 bp. CES4A4 = 780 bp.

4.2 Protein Expression of Human CES Isoforms

For the assessment of enzymatic activity and for live cell imaging, CES plasmids were used for transfected of Cos7 cells to express recombinant proteins. A pcDNA4/HisMaxC vector with an n-terminal His6 tag for enzyme assays and a pECFP-C1 vector with an n-terminal CFP tag for cell imaging experiments were used for the CES4A isoforms. In addition, constructs of CES1A3 tagged with N-term HIS6 (pcDNA4/HisMaxC) and CES2A1 and CES3A2 with Flag tag (pCI-neo) were used (provided by Manuel Tomisser). Cells were transfected, incubated for 48 hours, harvested and then cell lysate were prepared. To check for correct size of expressed protein (pcDNA4/HisMaxC and pCI-Neo constructs), Western blot was performed using an anti-flag AB coupled with horseradish peroxidase and mouse anti-his-AB in combination with anti-mouse IgG AB coupled with horseradish peroxidase. **Figure 5** shows all six expressed CES isoforms with either His6 or Flag tag. Bands for all isoforms were found at their approximate molecular weight including the weight of the tags (CES1A3: 67 kDa, CES2A1: 67 kDa, CES3A2: 67 kDa, CES4A1: 53 kDa, CES4A3: 53 kD, CES4A4: 43 kDa).

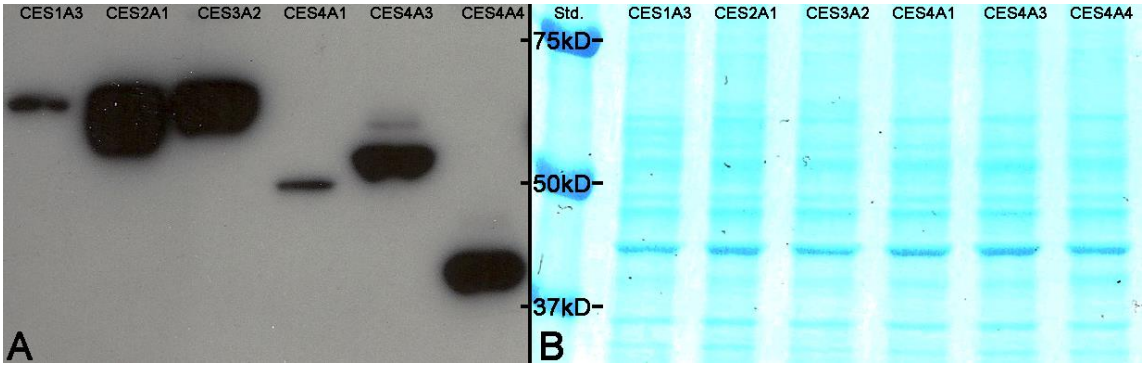

Figure 5: Western blot of CES isoforms expressed in Cos7 cells. For expression of recombinant proteins (CES isoforms), Cos7 cells were transfected with expression vectors encoding for His6 and Flag tagged CES isoforms. After incubation for 48h the cells were harvested and lysed in HSL-solution. 10 µg protein lysate protein were prepared with SDS-loading buffer, and separated on an acrylamide gel and blotted on a PVFD membrane. Recombinant proteins were examined by Western blot analysis. Anti-nterm-His6 AB with anti-mouse-HRP AB and anti-flag-HRP AB were used for 6His and Flag tag detection of recombinant proteins, respectively. Precision Plus Protein All Blue (Bio-Rad Laboratories, Hercules, USA) standard was used. (A) Western blot of recombinant CES isoforms. (B) Membrane stained with Coomassie brilliant blue.

4.3 Enzyme Assays

4.3.1 Retinyl Ester Hydrolyse Assay

To test the human CES isoforms for REH activity, RP was used as substrate. This assay was performed under 3 different conditions. A substrate containing Chaps, one containing PC and one containing both as detergents. The substrates were incubated with the different CES isoforms and LacZ-containing cell lysates and the free retinol was measured by HPLC. The amount of retinol indicates the activity of the enzymes for that substrate. **Figure 6** shows the REH activity of CES isoforms containing lysates with Chaps. In comparison to LacZ-containing lysates, CES1A3 and CES2A1-containing lysates showed significant activity for RP as substrate. CES4A4-containing lysates showed low but significant activity. For the other lysates, no activity was determined. If the substrate contained no Chaps and only PC for substrate presentation, all CES containing lysates showed turnover of RP (**Figure 7**). Basal endogenous activity of LacZ-containing lysates was very high in this experiment. For the last condition, RP emulsified with PC and Chaps, the basal activity of LacZ-containing lysates dropped down to the level of the first condition (**Figure 8**). CES1A3 and CES2A1-containing lysates showed significant activity but on a much lower level than with Chaps only.

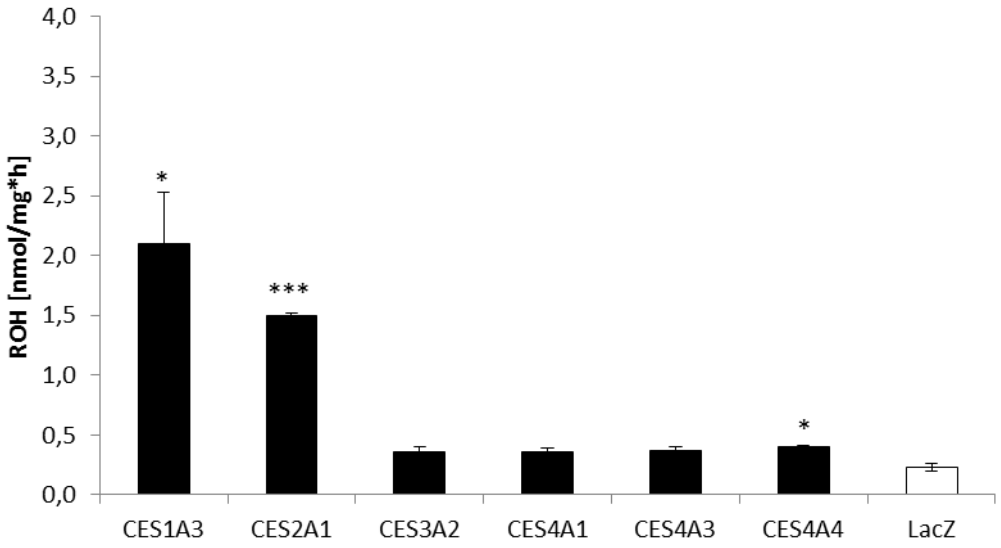

Figure 6: REH activity for CES isoforms with Chaps. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing RP and Chaps for 1 h at 37°C. The reaction was stopped and free retinol was extracted. Amount of free retinol was detected by HPLC. LacZ-containing lysates were used for endogenous activity valuation. Statistical significance was determined using Student's two-tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: * = $p<0.05$, *** = $p<0.001$

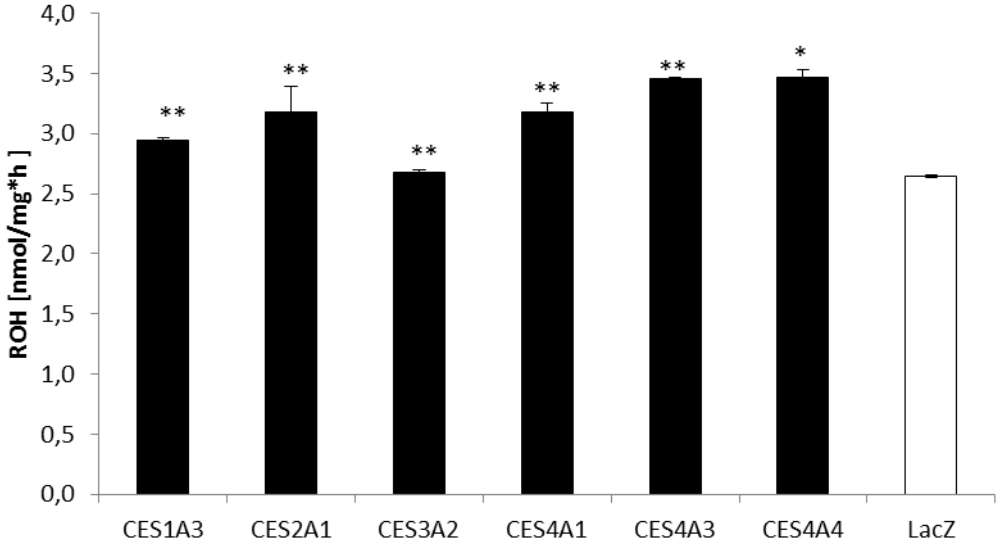

Figure 7: REH activity for CES isoforms with PC. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing RP and PC for 1 h at 37°C. The reaction was stopped and free retinol was extracted. Amount of free retinol was detected by HPLC. LacZ-containing lysates were used for endogenous activity valuation. Statistical significance was determined using Student's two-tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: * = $p<0.05$, ** = $p<0.01$

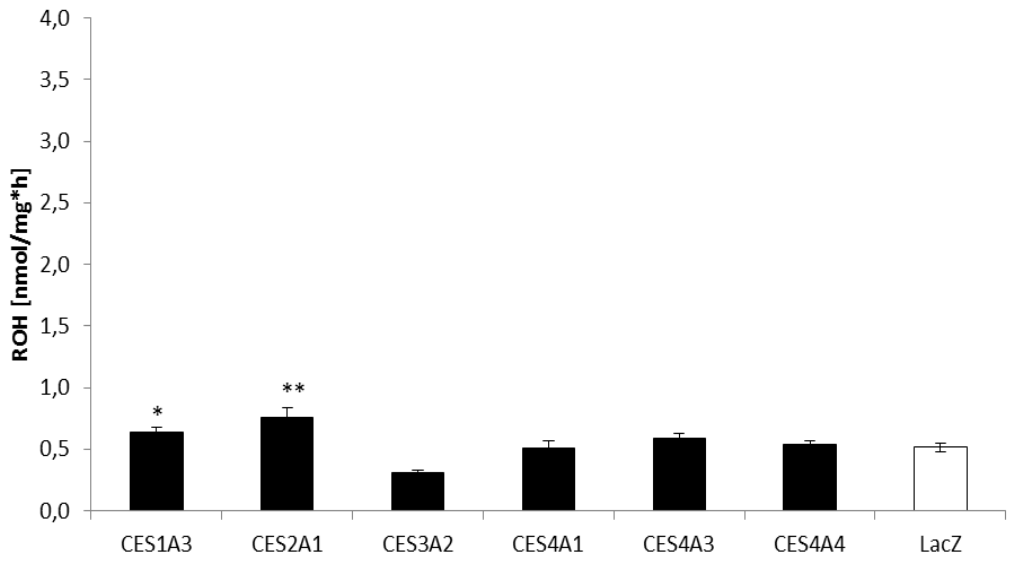

Figure 8: REH activity for CES isoforms with PC and Chaps. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing RP, PC and Chaps for 1 h at 37°C. The reaction was stopped and free retinol was extracted. Amount of free retinol was detected by HPLC. LacZ-containing lysates were used for endogenous activity valuation. Statistical significance was determined using Student's two tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: * = $p<0.05$, ** = $p<0.01$

4.3.2 Cholesteryl Ester Hydrolase Assay

To evaluate CES isoforms for CEH activity, lysates containing recombinant CES isoforms and LacZ protein were used and incubated with CO as substrate containing ^{3}H labelled CO and Chaps. After substrate incubation with CES isoforms or LacZ-containing cell lysates, the reaction was stopped and free cholesterol was extracted. Radioactivity was measured and CO hydrolysis calculated. The results, as displayed in **Figure 9**, showed high substrate turnover of CES1A3 (14 fold over LacZ) and CES2A1-containing lysates. CES4A4-containing lysates showed only slight hydrolysis with not much difference to the other isoforms and LacZ-containing lysates endogenous activity.

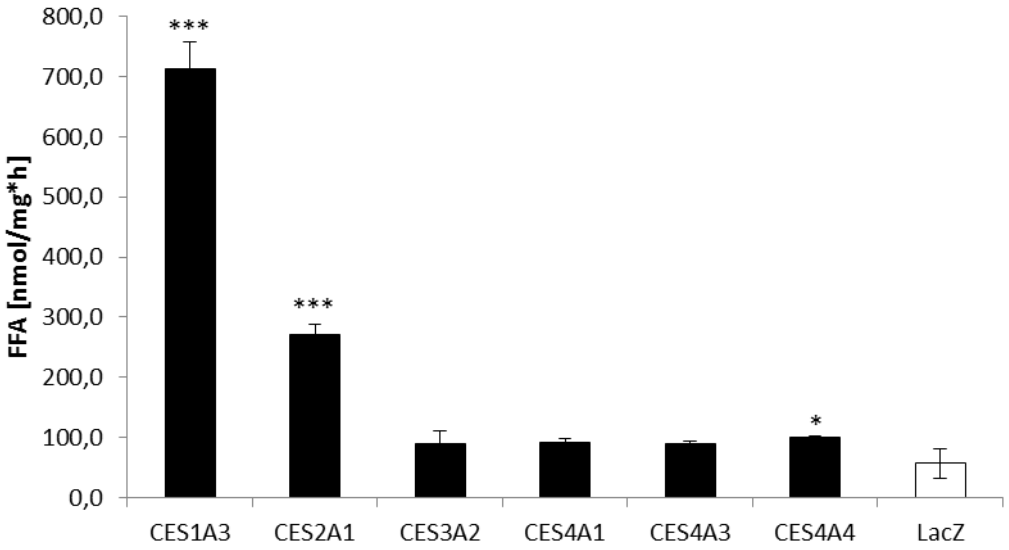

Figure 9: CEH activity for CES isoforms in a Chaps rich environment. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing ^{3}H-labelled CO and Chaps for 1 h at 37°C. The reaction was stopped and free cholesterol was extracted. Using scintillation counting the amount of free cholesterol was measured and the activity of lysates calculated. LacZ-containing lysates were used for endogenous activity valuation. Statistical significance was determined using Student's two tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: * = $p<0.05$, *** = $p<0.001$

4.3.3 Triacylglycerol Hydrolase Assay

The experimental objective was to determine the enzyme activity for TAG's of CES isoforms under two conditions. On the one hand, TO was emulsified with PC/PI and on the other hand with PC/PI and Chaps as additives. ^{3}H-labelled TO was used as tracer. After incubation of CES isoforms and LacZ-containing cell lysates with the two different substrates, the reaction was stopped, free FA extracted and labelled FA counted by liquid scintillation counting. **Figure 10** shows the activity of all CES isoform containing lysates for the substrate containing both, PC/PI and Chaps. CES1A3 and CES2A1-containing lysates showed TGH activity with 4 fold and 2.5-fold over LacZ-containing lysates, respectively. CES3A2 and CES4A3-containing lysates exhibited activity, however, very close to the endogenous LacZ-containing lysates activity. Other isoform containing lysates did not show any activity. In the absence of Chaps (**Figure 11**) the amount of TO hydrolysis drastically decreased. Except for CES3A2, all CES isoforms still showed significant activity but at a very low level.

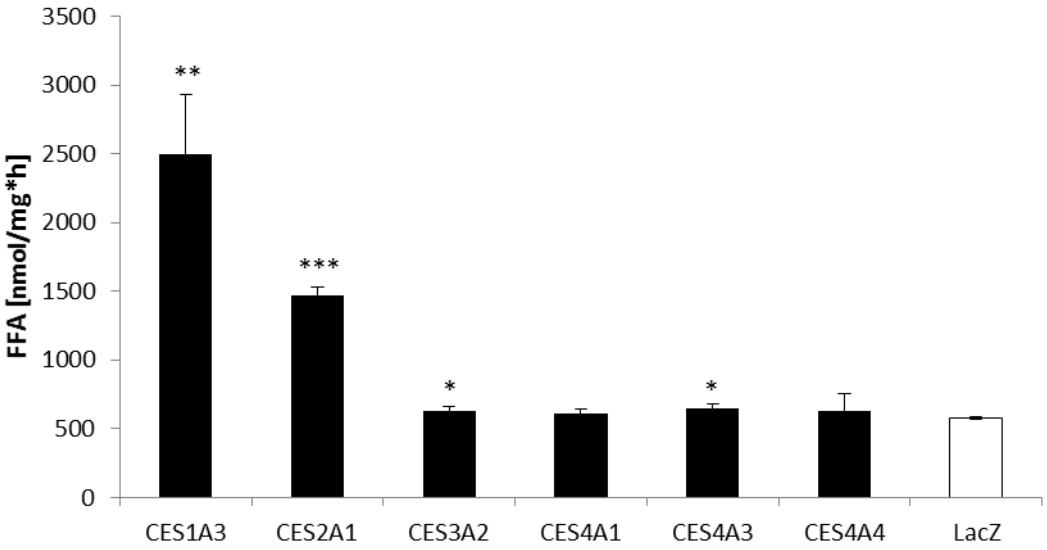

Figure 10: TGH activity of CES isoforms in PC/PI and Chaps containing conditions. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing ^{3}H-labelled TO, PC/PI and Chaps for 1 h at 37°C. The reaction was stopped and free FA were extracted. Using scintillation counting, the amount of free FA was measured and lysate activity calculated. LacZ-containing lysates were used for endogenous activity valuation. Statistical significance was determined using Student's two tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

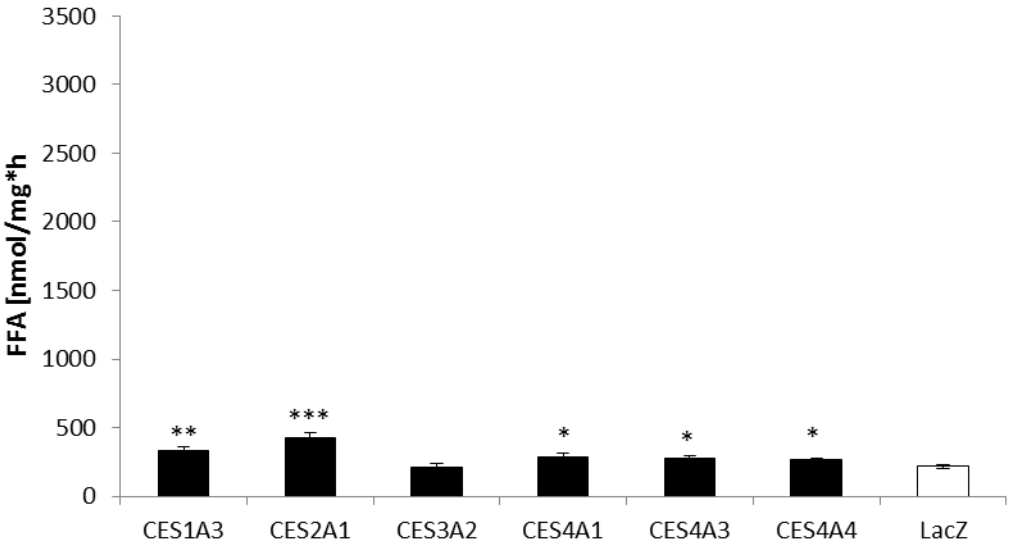

Figure 11: TGH activity of CES isoforms in PC/PI containing conditions. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing ^{3}H-labelled TO and PC/PI for 1 h at 37°C. The reaction was stopped and free FA were extracted. Using scintillation counting the amount of free FA was measured and lysate activity calculated. LacZ-containing lysates were used for endogenous activity valuation. Statistical significance was determined using Student's two tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

4.3.4 Triacylglycerol Hydrolase Assay – Additives Comparison

The experimental objective was to compare different forms of substrate additives regarding their influence on CES1A3 and CES2A1 TGH activity. A TGH activity assay with TO and ^{3}H-labelled TO was performed including three different additives in three different substrates: PC/PI, Chaps and Triton-X-100. For this test, only CES isoform containing lysates with the highest activity were used; CES1A3 and CES2A1. After incubation of the respective lysates with the different substrates for one hour, the reaction was stopped and free FA extracted. With liquid scintillation counting, the amount of free FA could be measured and the activity calculated. To evaluate the different conditions with each other, the results are shown in fold LacZ endogenous activity. For both CES1A3- and CES2A1-containing lysates, the highest activity was reached with the addition of Chaps (**Figure 12**). Under both PC/PI and Triton-X-100 conditions, CES1A3- and CES2A1-containing lysates showed activity at nearly the same level as LacZ endogenous activity.

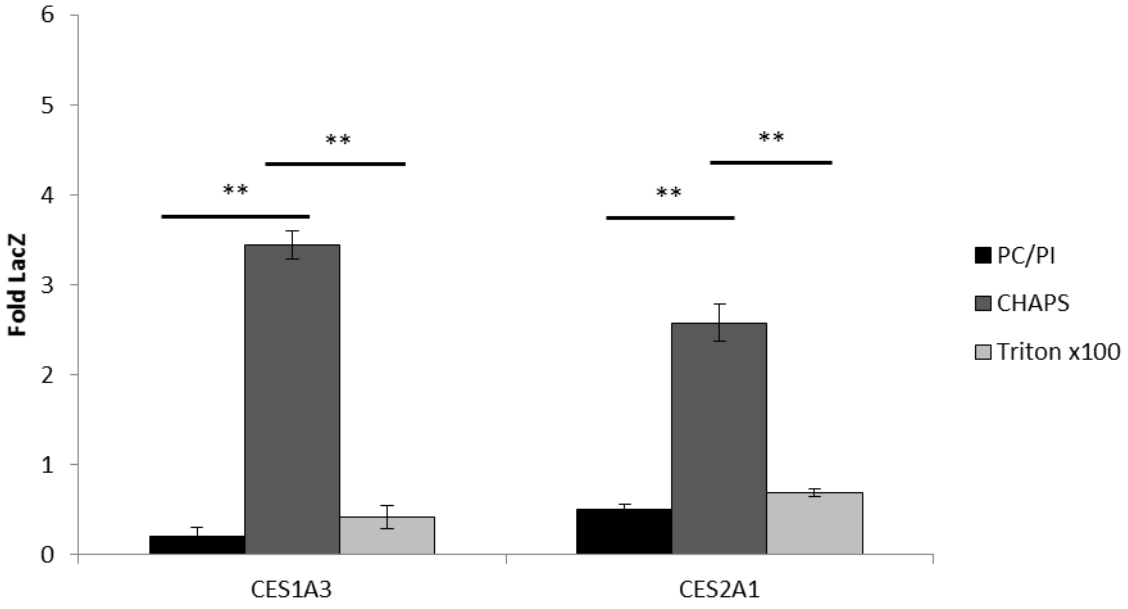

Figure 12: TGH activity comparison of CES1A3 and CES2A1 with PC/PI, Chaps and Triton-X-100 in fold LacZ. CES1A3 and CES2A1-containing cell lysates were incubated with ^{3}H-labelled TO for one hour. Each incubation was performed with 3 different additives: PC/PI, Chaps and Triton-X-100. After incubation, the reaction was stopped and the free FA were extracted. Using scintillation counting, the amount of free FA was measured and the TGH activity calculated. To compare the effect of the three additives, the results are depicted as fold LacZ lysate activity. Statistical significance was determined using Student's two-tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: ** = $p<0.01$

4.3.5 Monoglyceryl Ester Hydrolase Assay

Next, we tested CES isoforms for MGH activity. Using racOG as substrate, two conditions were tested, one with Chaps and one without. After incubation of the substrate with the lysates for 20 min, free FA were measured using the NEFA-kit and the MGH activity was calculated. **Figure 13** shows CES containing lysates MGH activity in the presence of Chaps. CES1A3 and CES2A1-containing lysates showed high enzymatic activity, with CES1A3 being the most active. The other isoforms showed no difference as compared to that of LacZ-containing lysate. The experiment performed in the absence of Chaps showed similar results (**Figure 14**). CES1A3 and CES2A1-containing lysates showed the highest enzymatic activity again, but on a much lower level. All other isoforms showed no or only slight activity compared to endogenous levels of LacZ-containing lysates.

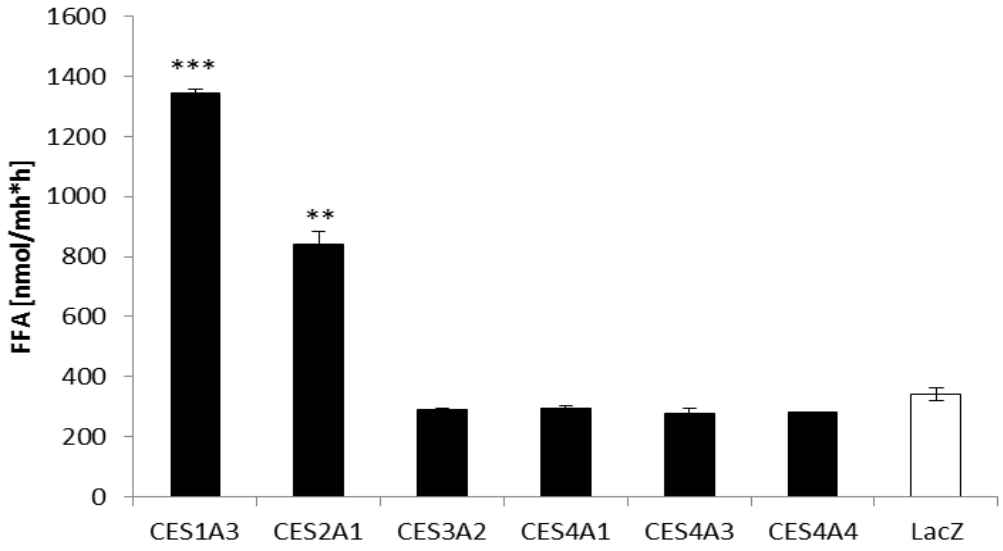

Figure 13: MGH activity of CES isoforms with Chaps. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing racOG and Chaps for 30 min at 37°C. Using the NEFA-kit, the amount of free FA was determined photometrically at 562 nm. LacZ-containing lysates were used for endogenous activity valuation. Statistical significance was determined using Student's two-tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: ** = $p<0.01$, *** = $p<0.001$

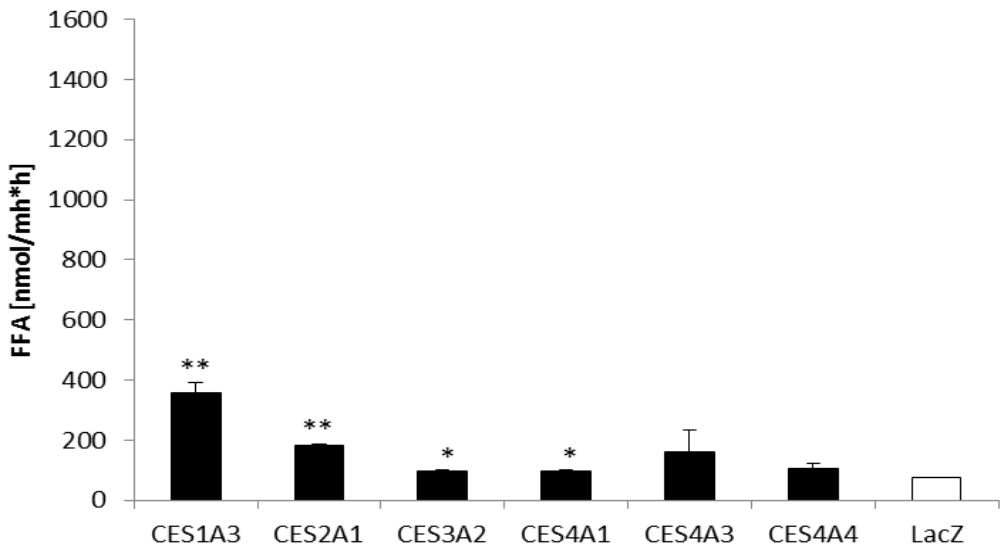

Figure 14: MGH activity of CES isoforms without Chaps. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing racOG for 30 min at 37 °C. Using the NEFA-kit, the amount of free FA was determined photometrically at 562 nm. LacZ-containing lysates were used for endogenous activity valuation. Statistical significance was determined using Student's two-tailed t-test. Data is presented as mean ± SD. Differences were considered significant at the following p-values: * = $p<0.05$, ** = $p<0.01$

4.3.6 p-Nitrophenylacetate-Assay

Aim of this experiment was to determine activity for the artificial substrate pNPA, which is water soluble and has a short ester bond. The substrate was prepared once with Chaps and once without. Both substrates were incubated with CES isoforms and LacZ-containing cell lysates for 5 min. Colour change was measured and indicated enzyme activity. As shown in **Figure 15** and **Figure 16**, activities with and without Chaps were the same. CES1A3 and CES2A1-containing lysates showed the highest and comparable activity under both conditions. All other isoforms showed no activity in both experiments, compared to LacZ-containing lysates. Statistical significance could not be calculated, as the sample size was only one.

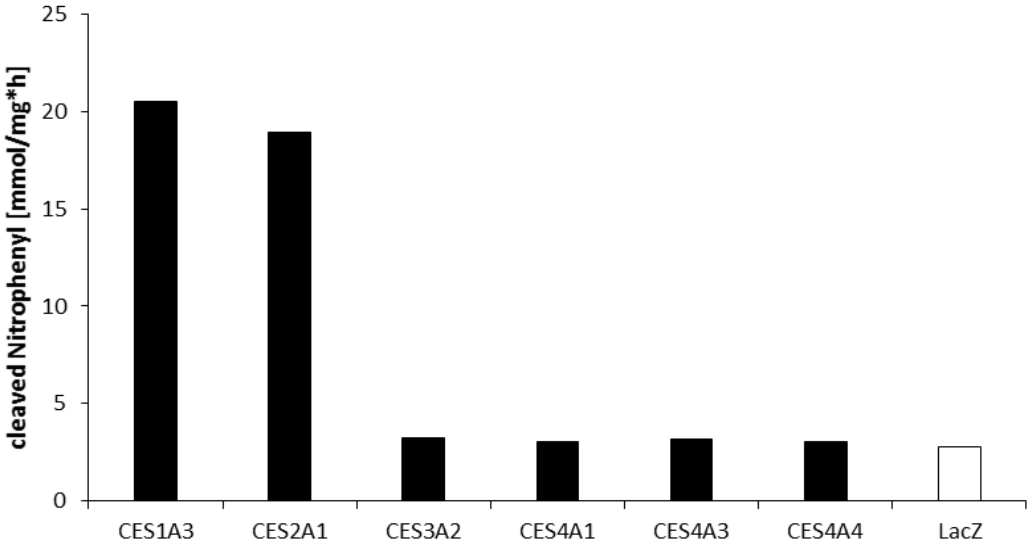

Figure 15: Enzyme activity of CES isoforms for pNPA with Chaps. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing pNPA and Chaps for 5 min at 37 °C. NPA turnover was measured photometrically at 562 nm and the enzyme activity calculated. LacZ-containing lysates were used for endogenous activity calculation. Statistical significance was not determined because the sample size was 1.

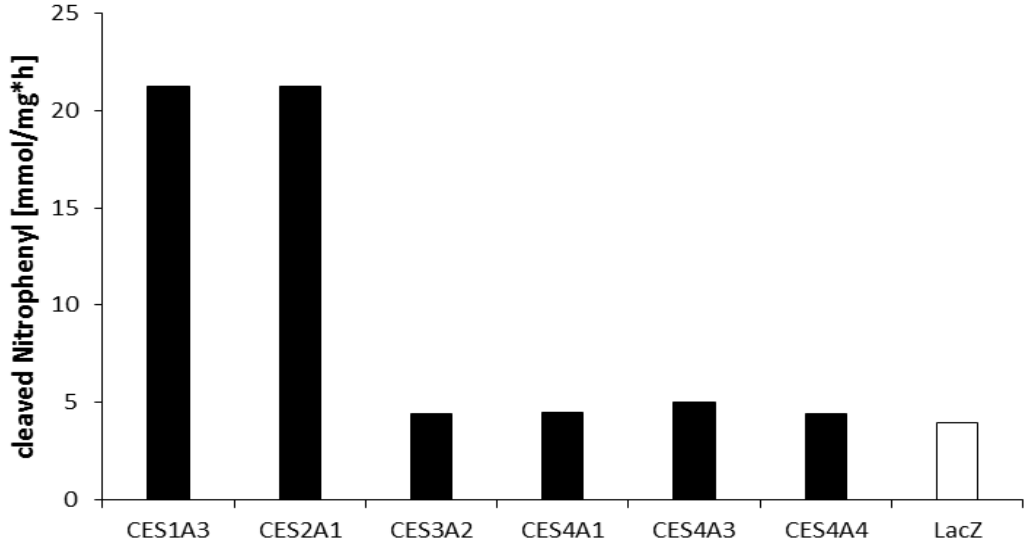

Figure 16: Enzyme activity of CES isoforms for pNPA. CES isoforms and LacZ-containing cell lysates were incubated with a substrate containing pNPA for 5 min at 37°C. NPA turnover was measured photometrically at 562 nm and the enzyme activity calculated. LacZ-containing lysates were used for endogenous activity calculation. Statistical significance was not determined because the sample size was 1.

4.3.7 Hit Finder

This assay was done to assess the hydrolytic activities of the CES4A isoforms for diglycerides as well as phospholipids including diphosphatidylglycerol. Substrates were prepared with Chaps and incubated with CES4A isoforms containing lysates. After one hour, free FA were measured and enzyme activity calculated. For the substrate phosphatidic acid, all CES4A isoform containing lysates showed significant hydrolytic activity. CES4A1-containing lysates showed significant activity for PE and CES4A4-containing lysates for NAPE. However, all lysates exhibited only marginal activity over the endogenous LacZ activity.

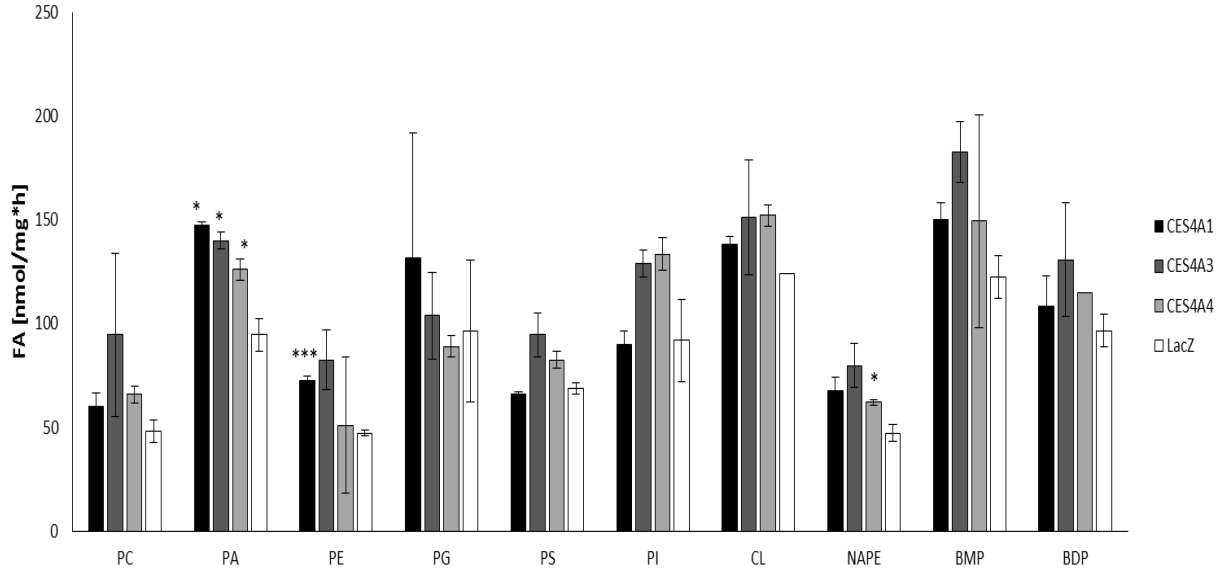

Figure 17: Hit Finder assay for CES4A isoforms with Chaps. CES4A isoforms and LacZ-containing cell lysates were incubated with various substrates (listed below) containing Chaps at 37 °C for 1 hour. FA release was measured using NEFA-kit and enzyme activity calculated. LacZ-containing lysates were used for endogenous activity calculation. PC = phosphatidylcholine, PA = phosphatidic acid, PE = phosphatidylethanolamine, PG = phosphatidylglycerol, PS = phosphatidylserine, CL = cardiolipin, NAPE = n-acyl-phosphatidylethanolamine, BMP = bis(monoacylglycero)phosphate, BDP = bis(diacylglycero)phosphate. Statistical significance was determined using Student's two-tailed t-test. Data are presented as mean ± SD. Differences were considered significant at the following p-values: * = $p<0.05$, *** = $p<0.001$

4.4 Live Cell Imaging

To investigate the cellular localisation of CES4A isoforms, live cell imaging was performed. Cos7 cells were transfected and grown on microscope cover slips, which were then imaged with a fluorescence laser-scanning microscope. In addition to the CES4A isoforms, CES1A3 was transfected and used as control, since its ER localisation within cells has been reported (12). Three different cell organelle markers were used for localisation analyses.

Figure 18 to Figure 21 show the microscope pictures of CES isoforms expression with ER, LD and lysosome markers in single and merged form. Experiments with CES1A3 showed expression in intracellular reticular patterns. Merging CES1A3 signals with ER-tracker signals

indicated localisation at the ER (**Figure 18**). CES4A1 displayed similar ER-like structures but only partial co-localisation with ER-tracker. Furthermore, defined reticular expression patterns of CES4A1 and ER-tracker collapsed if both were expressed in the same cell leading to aggregation of the signals (**Figure 19**). CES4A3 showed expression in medium to small sized intracellular aggregate-like structures with the highest density at close proximity to cell nuclei. Merging with ER, LD or lysosome tracker doesn't indicate localisation to those organelles (**Figure 20**). CES4A4 showed signals in close proximity to the cell nucleus with partial reticular structures (**Figure 21**). ER-tracker expression patterns collapsed and signals aggregated when co-expressed with CES4A4. No indication for localisation at LDs or lysosomes was found.

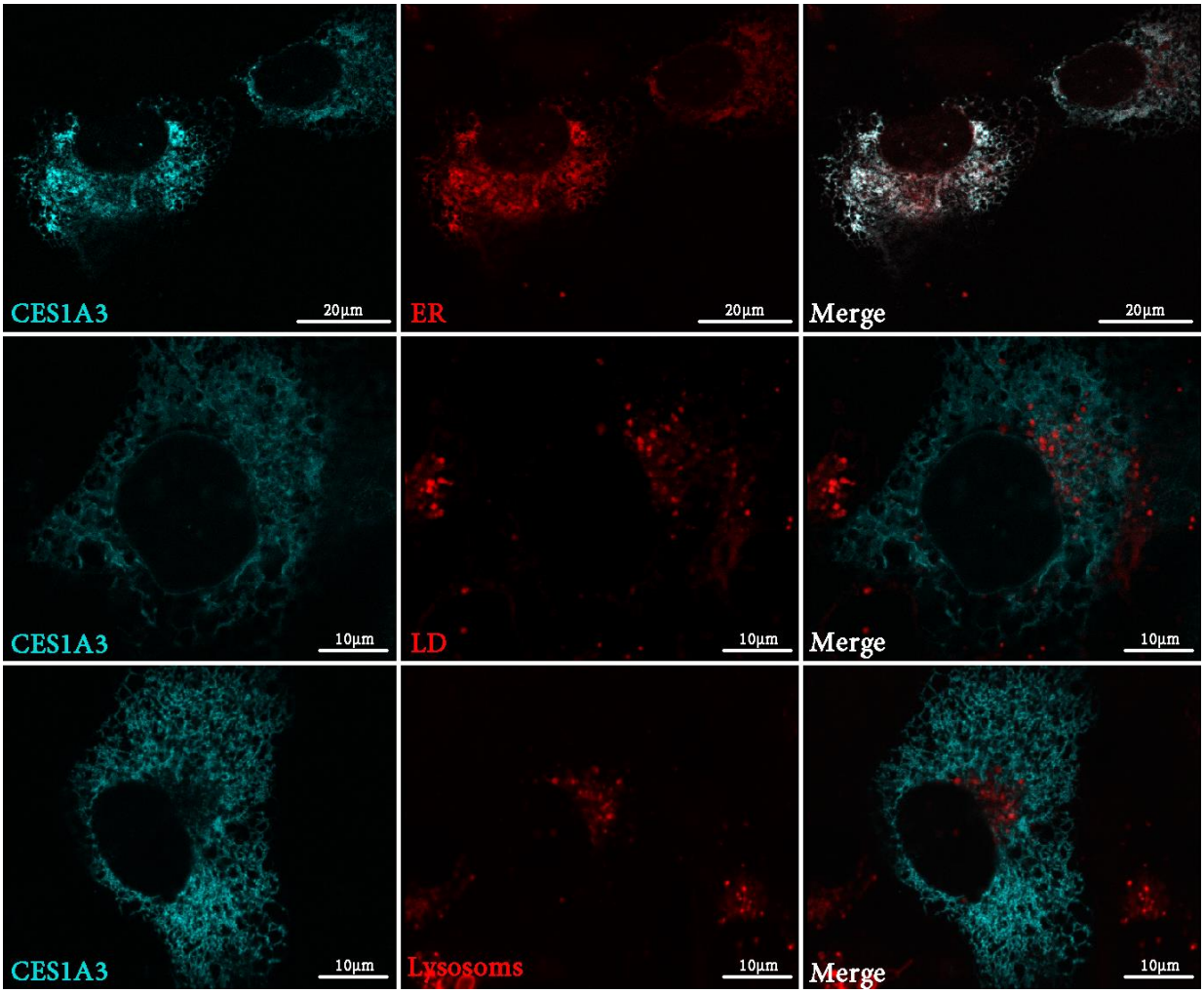

Figure 18: Live Cell Imaging of CES1A3 transfected Cos7 cells with ER-, LD- and lysosome- Tracker. Cos7 cells were grown and transfected with CFP-CES1A3 plasmid and ER-tracker. After 48h, the cells were incubated for one hour with LD- and Lysosome- tracker, respectively. Detection of the CFP and RFP signals was done by laser-scanning microscopy (Leica TCS SP2, Images taken by Wolinski H., University of Graz).

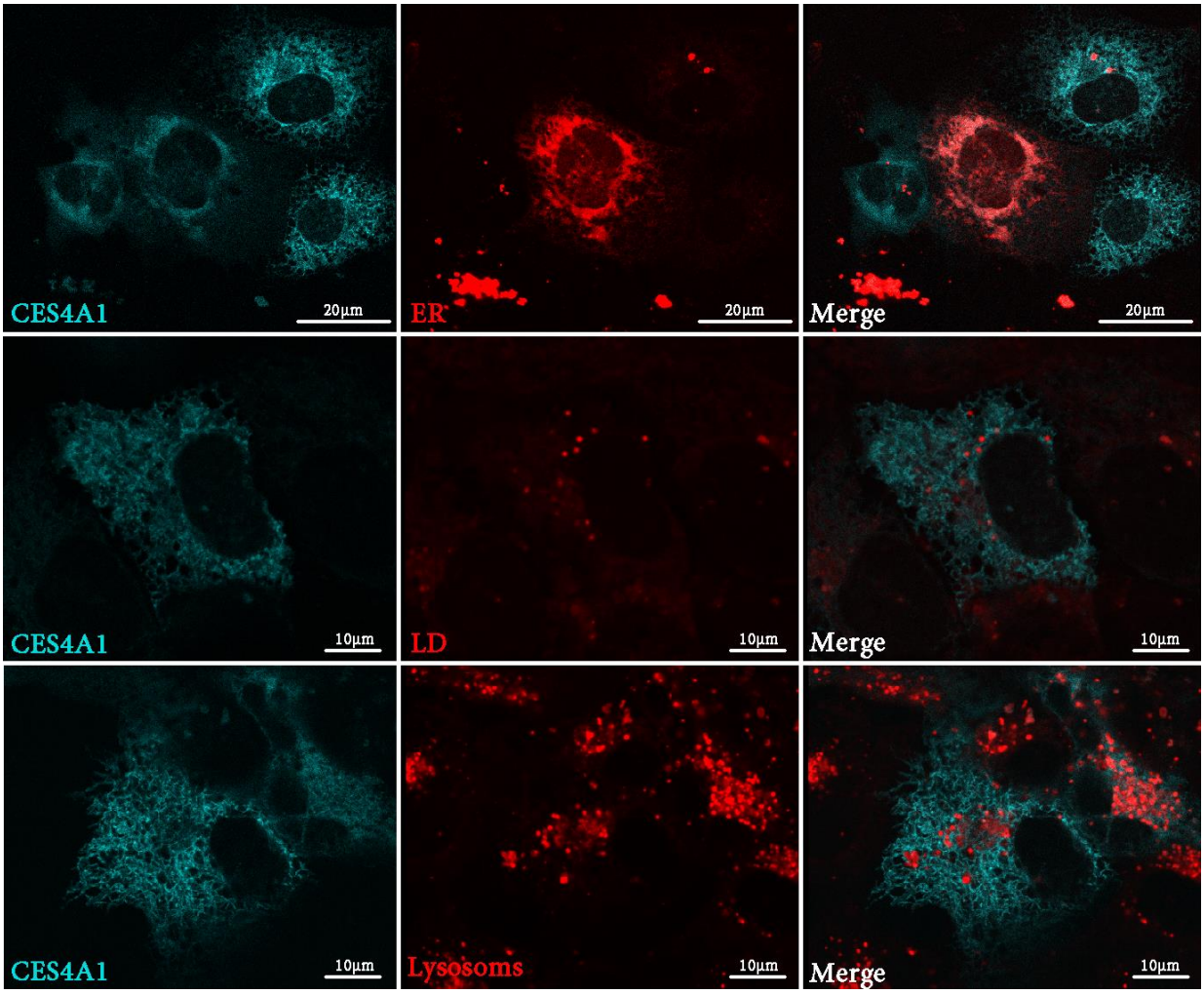

Figure 19: Live Cell Imaging of CES4A1 with ER-, LD- and lysosome- Tracker. Cos7 cells were grown and transfected with CFP-CES4A1 plasmid and ER-tracker. After 48h, the cells were incubated for one hour with LD- and Lysosome- tracker, respectively. Detection of the CFP and RFP signals was done by laser-scanning microscopy (Leica TCS SP2, Images taken by Wolinski H., University of Graz).

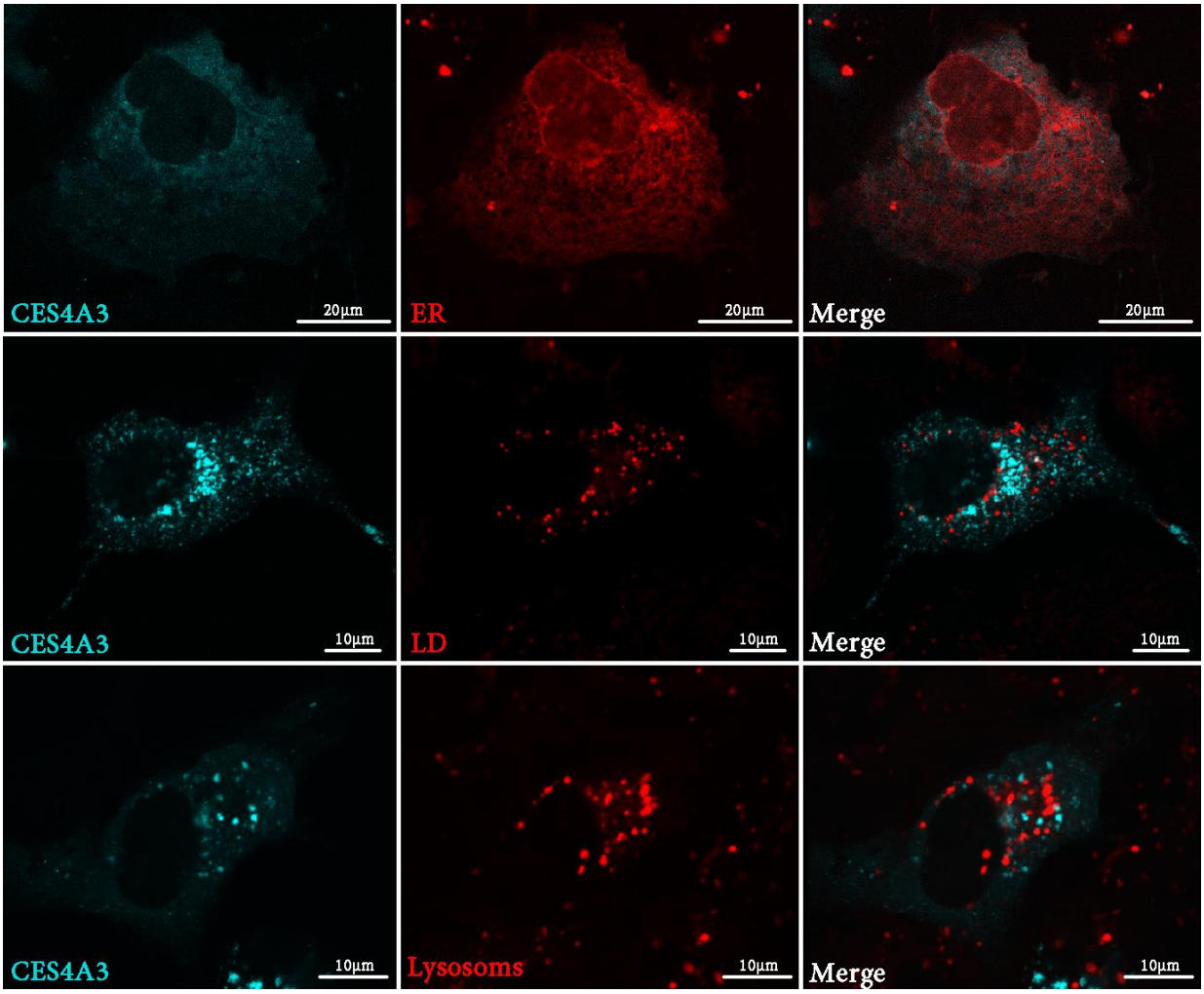

Figure 20: Live Cell Imaging of CES4A3 with ER-, LD- and lysosome- Tracker. Cos7 cells were grown and transfected with CFP-CES4A3 plasmid and ER-tracker. After 48h, the cells were incubated for one hour with LD- and Lysosome- tracker, respectively. Detection of the CFP and RFP signals was done by laser-scanning microscopy (Leica TCS SP2, Images taken by Wolinski H., University of Graz).

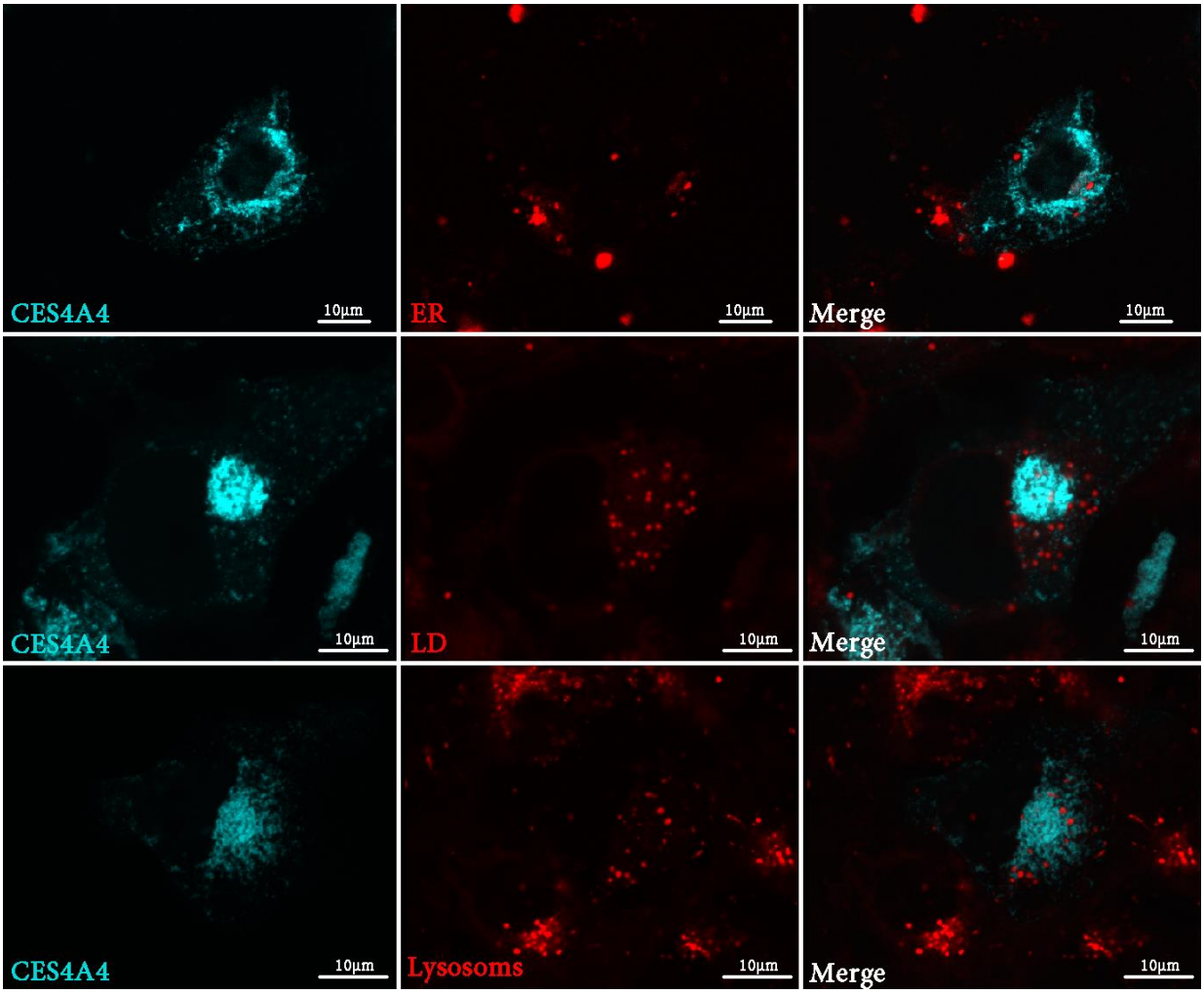

Figure 21: Live Cell Imaging of CES4A4 with ER-, LD- and lysosome- Tracker. Cos7 cells were grown and transfected with CFP-CES4A4 plasmid and ER-tracker. After 48h, the cells were incubated for one hour with LD- and Lysosome- tracker, respectively. Detection of the CFP and RFP signals was done by laser-scanning microscopy (Leica TCS SP2, Images taken by Wolinski H., University of Graz).

5 Discussion

The aim of this thesis was to investigate the endogenous roles and characteristics of the different isoforms of the human carboxylesterase family (CES5A isoforms weren't investigated due to lack of amplification). This should be achieved by analysing the enzymatic activity of the different isoforms, the conditions they need to be active as well as determining the localisation of the enzyme within cells.
The results of this work showed that CES1A3 and CES2A1 are active enzymes with hydrolytic activity for various lipid substrates such as MAG, CE, RE and TAG. Experiments with the use of different detergents for the substrate preparation showed that both isoforms exhibit highest activity in the presence of Chaps, a bile acid-like detergent. Other CES members, like CES3A2 and CES4A isoforms, exhibited only marginal to no activity for endogenous as well as artificial substrates, in the presence and absence of Chaps. Experiments with fluorescence labelled proteins showed localisation of CES1A3 at the ER. Similarly, CES4A1 also showed ER localisation. For CES4A3 and CES4A4, aggregation in small clusters around the nucleus with sometimes reticular structures was observed. There was no indication for lysosomal or LD localisation.

The aim of this thesis was to elucidate the endogenous enzymatic characteristics and location of different human CES isoforms. Most studies performed in the past focussed on drug and xenobiotic metabolism associated with CES. However, more recent studies indicate involvement of CES isoforms in various processes like TAG hydrolysis in adipocytes of WAT (13) and CE retention in human macrophages (14).
The first goal was to determine enzymatic activities of the human CES isoforms on endogenous esters with focus on neutral lipids. Cos7 cell lysates containing recombinant CES isoforms were prepared and used to perform different enzyme assays. Results of these experiments showed elevated hydrolytic activity of CES1A3 and CES2A1 expressing cells compared to LacZ expressing cells. Both lysates exhibited RP, CO, MAG and TAG hydrolase activities. However, the highest increase of hydrolytic activities was observed for CO and RP. For CES1A3, basal activity was elevated 14-fold and 10.5-fold, and for CES2A1 5.3-fold and 7.5-fold, for CO and RP respectively. This massive increase in CE hydrolytic activity for CES1A3 further supports an already indicated function of CES1 in the CE turn over (14; 15; 16), whereas REH activity has not been reported so far. However, tissue expression and REH activity could indicate involvement in vitamin A metabolism. Enzyme assays with TAG and MAG as substrate showed activities with CES1A3 and CES2A1 expressing lysates as well. However, the fold increase for TAG and MAG as substrate was much lower. Expression of CES1A3 and CES2A1 in the cells led to a 4-fold and 2.5-fold increase, respectively, for MGH and TGH activities of lysates. Other

CES isoforms did not show significantly increased activities for CEH, REH, MGH or TGH. These results further confirm the possible role of CES1 in the lipolysis of TAGs in adipose tissue (17) and suggests a possible involvement of CES2 as well.
In addition to neutral lipids, assays with CES4A isoforms, using other endogenous lipids as substrates, like phospholipids, were performed in a substrate screening assay. However, only marginal to no increase in activity was detected.
Experiments with the artificial substrate pNPA showed similar patterns as the experiments with neutral lipids. CES1A3 and CES2A1 showed strong hydrolytic activity. However, for CES3A2 and the CES4A isoforms, no activity was detected.
The results of these assays showed that CES1A3 and CES2A1 have the ability to hydrolyse a variety of endogenous lipids. However, other CES isoforms didn't exhibit any significant hydrolytic activity with the tested substrates in this study. These findings indicate a role of CES1A3 and CES2A1 in the neutral lipid turn over.

It is important to notice that CES1A3 and CES2A1 displayed activity only when Chaps was added to the substrate (except for MGH and pNPA activity). Chaps is a zwitterionic detergent with high similarity to bile acid. This is especially interesting as CES1A3 and CES2A1 are mainly expressed in liver and intestine, with CES1A3 being most abundant in the liver but not the intestine and CES2A1 in the liver and intestine (18). These are organs with high bile acid concentrations (19). CES1 was also reported to be important in cholesterol elimination and bile acid synthesis by Bin Zhao et al (20). This could indicate a possible positive feedback loop for CEH activity of CES1. Furthermore, Bencharit et al (21) reported cholate binding capability of human CES1 and conformational change from its hexamer to its trimer state which could promote the hydrolytic activity of CES1. However, further experiments with Chaps and its possible CES activating properties need to be conducted to further investigate this topic.
Interestingly, CES1A3 and CES2A1 showed comparably high hydrolytic activity for MAG and pNPA as substrates, in the presence and absence of Chaps. Both of these substrates provide relatively easy excess to their ester bonds, which could explain the CES activity even in the absence of Chaps.
Notably, CES3 and CES4A isoforms didn't show any activities, even in the presence of Chaps. There are several possible reasons for that. It is possible that these enzymes require other assay conditions or show activity with other substrates. Furthermore, these isoforms could need special posttranslational processing not present in a Cos7 cells based expression system. It is interesting that these two CES groups show much lower mRNA levels compared to CES1 and CES2 (18) and could therefore be much less important. There are many possible reasons. Further experiments are needed to explore these possibilities.

Lastly, live cell imaging was performed to analyse localisation of the proteins in cells. Recombinant CFP tagged CES1A3 and CES4A isoforms were expressed in Cos7 cells and markers were used for different organelles. In these experiments, CES1 was used as control as its localisation at the ER is already known (12). The results showed different locations for each CES4A isoform. CES4A1 strongly indicates ER localisation, but co-expression with ER marker was not possible. It seems that if both marker and CES4A1 are co-expressed, only one of them locates correctly. This conclusion was drawn as either protein always showed ER-like structures in the absence or clustering of the other. But even without marker co-localisation, fluorescence signals showed ER-typical structures. For CES4A3 and CES4A4 no explicit location was found as no co-localisation with markers for ER, LDs or lysosomes was observed. CES4A3 showed clustering of different sizes within the cell, with very small clusters when co-transfected with ER marker and medium sized cluster with LD and lysosome makers (but no marker co-localisation). One could speculate that those clusters are early endosomes/exosomes. However, further experiments are needed to investigate this hypothesis. CES4A4 localisation studies delivered no distinct results. Signals with reticular ER-like structures as well as clusters were observed. Those signals were found near the nucleus. However, no co-localisation with ER, LD and lysosome marker was observed. Co-expression with ER marker proteins resulted in clustering of either ER marker or CES4A4, similar to CES4A1. Localisation at the ER near the nucleus would be plausible but was not explicitly confirmed and would need further experiments.

In conclusion, results of this thesis show that members of the human CES family are able to hydrolyse not only xenobiotic substrates but endogenous lipid ester substrates as well. It shows that especially CES1 and CES2 are able to hydrolyse a wide variety of substrates like REs, CEs, MAGs and TAGs whereas CES3 and CES4 only show marginal to no activity. Furthermore, it was observed that Chaps, a bile acid-like detergent, is crucial for the activity of CES family members when using hydrophobic substrates. Localisation experiments for CES1 confirmed the published localisation within the ER. However, experiments with CES4 isoforms didn't show clear results. CES4A1 localisation at the ER is likely but could not be verified by co-localisation with marker proteins. The results strongly indicate potential involvement of at least CES1 and CES2 in human endogenous lipid ester hydrolysis. Furthermore, the results deliver more evidence for the role of CES1 in CE and TAG metabolism.

In future experiments, the role of the human CES family on endogenous substrates should be further elucidated. As CES isoforms seem to be very versatile enzymes, they could be important in many not yet established pathways. One major focus in future studies should be to further investigate the importance of Chaps or bile acid on the function of CES family members. Chaps titration assays would be highly interesting to determine activating properties of Chaps on

human CES isoforms. Furthermore, lipidomic analysis of overexpressing/silencing CES isoforms in cells could yield important information on the role of different CES family members in various cell types. Additionally live cell imaging experiments are necessary to potentially confirm ER localisation and to clarify the localisation of CES4A3 and CES4A4.

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