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1 Abstract

In recent years, research on phospholipases has turned increasingly away from phenomenology and regulation and has moved into the molecular cell biology of the structure and function of its isoenzymes in eukaryotic cells. Phospholipases are grouped into the main classes A_1, A_2, B, C and D. The classification depends on the site of hydrolysis at the sn-1 or sn-2 acyl ester bond, at the glycerol phosphate bond or at the glycerol phosphate-basephosphodiester bond. The resulting products of catalysis depending on the specific enzyme and substrate include free fatty acids, lysophospholipids, diacylglycerol, phosphatidic acid and phosphorylated or free base. These products are frequently found to have second messenger activity and therefore play a major role in regulatory processes. Eicosanoids are lipid mediators derived from phospholipase-released arachidonic acid that are involved in numerous homeostatic biological functions as well as in a number of disease states as inflammation. PGE_2 is the main prostaglandin produced in bone tissue and is synthesized and secreted by osteoblastic cells.

In this thesis we tried to elucitade the correlation between phospholipase D1, D2 and cytosolic phospholipase A_2 in endothelin-1 induced arachidonate liberation and prostaglandin E_2 formation by osteoblast-like MC3T3-E1 cells. Experiments were performed using small interfering RNA duplexes (siRNA) to silence one of those PLAs, to examine which of the three phospholipases is directly responsible for arachidonic acid liberation and prostaglandin E_2 formation. The latter is to be identified by means of negative chemical ionisation, the mass spectrum and the retention time. Quantification took place by using D_4-PGE_2 as an internal standard. The success of silencing was examined by Western Blot and PCR.

Based on this, we have shown that independently from PLD2 and PLD1, $cPLA_2$ is the big player in AA release and PGE_2 formation. As well as the addition of Ni^{2+} increases PGE_2 production, which is known as the “nickel effect”.[1,2] By the means of our experiments, the role of PLD1 and PLD2 can be neither précised or confirmed nor refused. Continued experiments on this matter need to include the improvement of clean antibodies for Western Blot and a qPCR method with higher sensitivity and specificity.

[1] {Leis 1994 #23}
[2] {Leis 2016 #24}

2 Kurzzusammenfassung

In den letzten Jahren hat sich die Forschung in eukaryotischen Zellen, zunehmend der molekularen Zellbiologie, sowie der Struktur und Funktion der Phospholipasen und ihren Isoenzymen zugewandt. Phospholipasen werden in die Hauptklassen A_1, A_2, B, C und D unterteilt. Die Klassifizierung hängt von der Stelle der Hydrolyse an der sn-1 oder sn-2 Acylesterbindung, an beiden Esterbindungen oder vor beziehungsweise nach dem Phosphoratom. Die resultierenden Produkte der Katalyse sind abhängig von Enzym und Substrat und umfassen freie Fettsäuren, Lysophospholipide, Diacylglycerin, Phosphatidsäure und phosphorylierte oder freie Base. Diese Produkte besitzen Second-Messenger-Aktivität und spielen daher eine wichtige Rolle bei regulatorischen Prozessen. Eikosanoide sind Lipidmediatoren, die durch von Phospholipasen freigesetzte Arachidonsäure entstehen. Sie sind an zahlreichen biologischen, homöostatischen Funktionen sowie an einer Reihe von Krankheitszuständen wie Entzündungsprozessen beteiligt. PGE_2 zählt zum Hauptvertreter dieser Gruppe, welches im Knochengewebe erzeugt und synthetisiert, und von osteoblastische Zellen sezerniert wird.

Zielsetzung dieser Masterarbeit war es eine Korrelation zwischen den Phospholipasen D1, D2 und zytosolischer Phospholipase A_2, bei der Endothelin-1-induzierten Arachidonat-Freisetzung und daraus resultierender Prostaglandin E_2-Bildung in MC3T3-E1-Zellen zu finden. Die Experimente wurden unter Verwendung von kurzen RNA Doppelsträngen (siRNA), um die Expression der spezifischen Phospholipasen zu unterdrücken, durchgeführt. Dadurch sollte herausgefunden werden, welches der drei Enzyme direkt an der Arachidonsäurefreisetzung und Prostaglandin E_2-Bildung verantwortlich ist. PGE_2 wurde mittels negativer chemischer Ionisierung, dem Massenspektrum und der Retentionszeit identifiziert. Die Quantifizierung erfolgte durch die Verwendung von deuteriertem PGE_2 als internem Standard. Der Erfolg der unterdrückten Genexpression wurde durch Western Blot und PCR überprüft.

Im Zuge der Masterarbeit konnte bestätigt werden, dass die cytosolische PLA_2 die hauptverantwortliche Phospholipase für die AA-Freisetzung und PGE_2-Bildung ist. Ebenso konnte durch Zugabe von Ni^{2+} die PGE_2-Produktion signifikant erhöht werden, was als "Nickel-Effekt" beschrieben wird. Welche Rolle PLD1 und PLD2 konkret dabei einnehmen konnte weder bestätigt oder präzisiert, noch widerlegt werden.

3 Abbreviations

AA	Arachidonicacid
AD	Alzheimer's disease
ADP	Adenosinediphosphate
AdPLA	Adiposephospholipase
BEL	Bromoenollactone
BSA	Bovine serumalbumin
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
Ca^{2+}	Calcium
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
$cPLA_2$	Cytosolicphospholipase A_2
CYP	Cytochrome P450
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DIPEA	N,N-Diisopropylethylamine
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ET	Endothelin
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GPCRs	G protein-coupledreceptors
GTP	Guanosine-5'-triphosphate
HPLC	High performance liquid chromatography
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
kDA	Kilodalton
LDS	Lithium dodecyl sulfate
$LPLA_2$	Lysosomalphospholipase A_2
LPP	Lipid phosphate phosphatase
lyso-PA	Lysophosphatidicacid
MAP kinase	Mitogen-activatedproteinkinases
MAPKAPK	MAPK-activatedproteinkinase
MC3T3-E1	Mouse calvaria-derived pre-osteoblastic cells
MEM	Minimum essential medium
MES	2-(*N*-morpholino)ethanesulfonic acid
MOX	Methoxamine
MS	Massspectrometry
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
NCBI	National Center for Biotechnology Information

NICI	Negative ionchemicalionization
NSAIDs	Nonsteroidal anti-inflammatorydrugs
PA	Phosphatidicacid
PAF-AH	Plateletactivatingfactoracetylhydrolase
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PFBBr	Pentaflourobenzyl bromide
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PH	Pleckstrin homology domain
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLA_2	Phospholipases A_2
PLAs	Phospholipases
PLD	Phospholipase D
PP	Polypropene vials
PX	Pbox consensus sequence domain
qPCR	Quantitative reverse transcriptase PCR
RIA	Radioimmunoassay
RT	Roomtemperature
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA duplexes
$sPLA_2$	Secretedphospholipase A_2
TBST	Tris-bufferedsaline
TMCS	Trimethylchlorosilane
vWf	von Willebrand factor

4 Introduction

4.1 Prostaglandins and inflammation

Inflammation in general is the body's immune system's response to stimulus. During the acute phase a rapid influx of blood granulocytes occurs, followed by monocytes that mature into inflammatory macrophages that subsequently proliferate and thereby affect the functions of resident tissue macrophages. When granulocytes are eliminated and macrophages and lymphocytes return to normal pre-inflammatory numbers and phenotypes, the outcome of this acute inflammation is resolution, repair and restore of tissue damage. The key role of the prostaglandins (PG) in this process is located in the generation of inflammatory response.

The production of those proinflammatory lipid mediators, the eicosanoids (*i.e.* prostaglandins and leukotrienes), is dependent on the availability of the precursor, free arachidonic acid (AA), a 20-carbon unsaturated fatty acid. The collectively termed prostanoids are formed when arachidonic acid is released from the plasma membrane by phospholipases (PLAs) and metabolized by the sequential actions of prostaglandin endoperoxide G/H synthase, or cyclooxygenase (COX), and respective synthases.

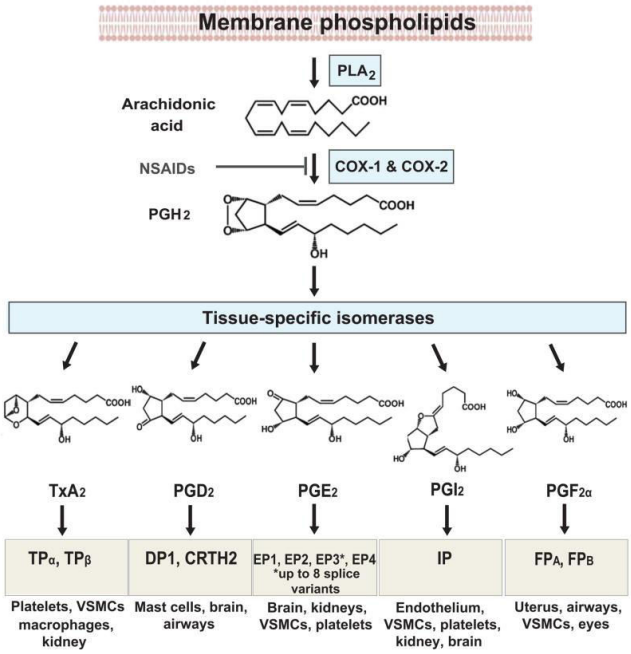

Figure 1: Biosynthetic pathway of prostanoids[3]

[3] {Ricciotti 2011 #32}

Phospholipase A_2 (PLA_2) enzymes play a central role in controlling the release of arachidonic acid. The mechanism of regulating the release of AA by the arachidonic acid-selective, 85-kDa cytosolicPLA_2 ($cPLA_2$) is now well recognized.

Prostaglandins are biosynthesised from AA when required, and are not normally stored in tissues.[4] If there is an extracellular hormonal stimulus they are synthesised and then released rapidly (5-60s).[5] Each cell type usually generates one or two dominant products but in general prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) are the four bioactive forms generated in vivo.[6] Prostaglandins exert their effects by activating G protein-coupled receptors (GPCRs).

Prostaglandins are well known to sustain homeostatic functions and mediating different mechanisms such as the inflammatory response.[7] Despite these facts, some studies suggest that prostaglandins have also pro-inflammatory effects. Until know their role in resolution of inflammation is more controversial. Those mechanisms for example were described in animal models where prostaglandin D and F synthases declined during the phase of acute inflammation, and levels increased during the resolution phase, suggesting their possible role in resolving inflammation. Due to that, it was mentioned that classic inhibitors of PG synthesis such as nonselective and cyclooxygenase-2 (COX-2) selective inhibitors (nonsteroidal anti-inflammatory drugs, NSAIDs) may actually prolong inflammation when administered during the resolution phase.[8,9]

4.1.1 Gas chromatography-mass spectrometry (GC-MS) of prostaglandins

Prostaglandins and related compounds can be detected and assessed by several methods such as radioimmunoassay (RIA), high performance liquid chromatography (HPLC) linked to RIA, UV or mass spectrometric detection. To date the combination of gas chromatography- mass spectrometry is the preferred method to quantify PG levels. This method combines the high resolution of the gas chromatographic separation using fused silica capillary columns with the specificity and sensitivity of mass spectrometry. For the quantification an internal standard with similar physicochemical properties to the compound being analysed, is essential for each measurement. Due to the fact that chemically untreated prostaglandins are not suitable for GC analysis, functional moieties need to be protected first to improve chromatographic separation and ensure the assay sensitivity and

[4] {Karim, S. M. M 1976 #18}
[5] {Ridgway #33}
[6] {Ricciotti 2011 #32}
[7] {Ricciotti 2011 #32}
[8] {Scher 2009 #39}
[9] {Sales 2003 #36}

specificity. These two properties are also characteristic for negative ion chemical ionization (NICI), thus has made it the method of choice for quantitative analysis of PGs.[10]

4.2 Phospholipases

Phospholipases comprise enzymes that catalyze the cleavage of phospholipids, grouped into the main classes, namely A_1, A_2, B, C and D (figure 2). These enzymes function at the interface with organized membranes. The classification depends on the site of hydrolysis at the sn-1 or sn-2 acyl ester bond, at the glycerol phosphate bond or at the glycerol phosphate-basephosphodiester bond, respectively.[11] The resulting products of catalysis depending on the specific enzyme and substrate include free fatty acids, lysosphospholipids, diacylglycerol, phosphatidic acid and phosphorylated or free base. These products are frequently found to have second messenger activity and therefore play a major role in regulatory processes like cell metabolism, proliferation, motility and apoptosis. But it is also well known that those lipid messengers are involved in pathological conditions like inflammation reactions.

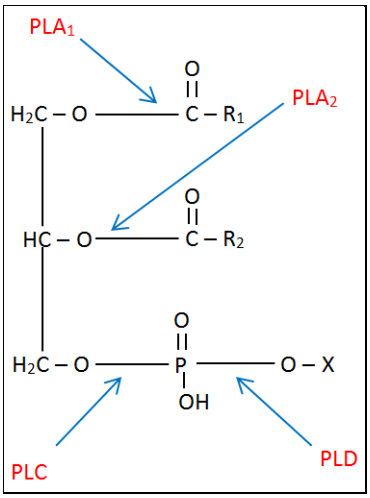

Figure 2: Sites of attack of phospholipases on their phospholipid substrate

Phospholipases are ubiquitous in all domains of life. They are found in distinct shapes such as cytosolic-, membrane bound- and secreted forms of the enzyme. Phospholipases differ greatly in their size, structure and substrate specificity. Due to that fact, they show a great diversity in their mode of action, regulation and physiological role. The cellular function from many of those enzymes has been examined using various techniques of molecular biology including gene transfection, gene knockout and antisense strategies.[12]

[10] {J. Nourooz-Zadeh and C. C. T. Smith 2000 #15}
[11] {Rosenthal 2009 #35}
[12] {Aloulou 2012 #2}

4.3 Phospholipase A_2 superfamily

The members of the phospholipase A_2 superfamily differ in their biological functions, three-dimensional structures as well as in their catalytic residues and specific tissue localization. The different PLA_2s were found to play a role in several cellular processes like lipid metabolism, membrane remodelling, inflammation and apoptosis. The superfamily consists of a set of enzymes that catalyze hydrolysis of the *sn*-2 ester bond of phospholipids. The *sn*-2 position of phospholipids frequently contains unsaturated fatty acids such as arachidonic acid.[13] AA is the precursor of the eicosanoids, which include prostaglandins and leukotrienes. The release of AA by phospholipase A_2 is the most straight forward way to increase the levels of free AA.[14]

To date, at least 19 enzymes that possess PLA_2 activity have been identified in mammals.[15] PLA_2s have been systematically classified on the basis of their nucleotide and amino acid sequence. Sorted into six main categories, namely secreted phospholipase A_2 ($sPLA_2$), Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$), Ca^{2+}-independent phospholipase A_2 ($iPLA_2$), platelet activating factor acetylhydrolase phospholipase A_2 (PAF-AH), lysosomal phospholipase A_2 ($LPLA_2$) and adipose phospholipase (AdPLA).[16]

Due to the fact that each of these enzymes is a key mediator in lipid metabolism and pathophysiological conditions, pharmaceutical and biotechnology industry have pursed the design of selective drugs with potential anti-PLA_2 effects.[17] Because of the fact that numerous PLA_2 enzymes with overlapping properties (e.g. site of expression and inhibition susceptibility) have been identified, it has proved problematic to target and inhibit the enzyme reaction.[18] Regarding to that characteristics, the direct inhibition of a PLA_2 enzyme could block all pathways, which are regulated by the PLA_2 reaction, at once.

4.3.1 Secreted phospholipase A_2

As the name suggests, the group of $sPLA_2$s are small secreted proteins of 14–18 kDa (except for Group III $sPLA_2$) that usually contain 6 to 8 disulfide bonds. [19] The first $sPLA_2$ was isolated from cobra venom, over 100 years ago. After studying various snake venom $sPLA_2$s, the first mammalian $sPLA_2$ was isolated from pancreatic bovine juice in 1977. Until now, 17 forms of those secreted phospholipases have been

[13] {Burke 2009 #5}
[14] {Balsinde 2002 #3}
[15] {Murakami 2002 #28}
[16] {Balsinde 2002 #3}
[17] {Burke 2009 #5}
[18] {Balsinde 2002 #3}
[19] {Schaloske 2006 #38}

identified, ten of them in mammals. Due to that wide range of isoforms, they differ in their tissue distribution patterns and physiological functions.[20]

The mammalian $sPLA_2$s are grouped and numbered according to their disulfide bonding patterns and in order of their discovery.[21] The secreted phospolipases in general are characterized by their molecular weight, which is lower than that of many others from the PLA_2 subclasses. A schematic illustration (table 1) provides an overview of these PLA_2 types. They contain a highly conserved catalytic domain and a Ca^{2+}(calcium) binding loop. According to the structural features this group of enzymes requires a micromolar level of Ca^{2+} for catalytic activation.[22] The enzymes are highly disulfide linked and histidine is found at the active site of the catalytic domain. Some members of the $sPLA_2$ group (II A, II D, II F, III, V and X) have been identified to trigger AA metabolism to produce pro-inflammatory mediators like prostaglandins, leukotriens and thromboxanes. Recent studies confirmed that $sPLA_2$s are involved in multiple steps in atherosclerosis as well as in pathological effects of adult respiratory stress syndrome, inflammatory bowel disease and pancreatitis.[23]

[20] {Dennis 2011 #8}
[21] {Schaloske 2006 #38}
[22] {Dennis 2011 #8}
[23] {Rosenson 2009 #34}

Table 1: Classification of secreted PLA_2 according to Dennis

group	Molecular weight [kDa]	disulfide bonds	source
I A	13-15	7	cobra/kraits
I B	13-15	7	human/porcine pancreas
II A	13-15	7	rattlesnake, human synovial
II B	13-15	6	gaboon viper
II C	15	8	rat/ murine testis
II D	14-15	7	rat/ murine pancreas/ spleen
II E	14-15	7	human/ murine brain/ heart/ uterus
II F	16-17	6	human/ murine testis/ embryo
III	15-18 55 (human/murine)	8	human/ murine/ lizard/ bee
V	14	6	human/ murine heart/ lung/ macrophages
IX	14	6	snail venom
X	14	8	human spleen/ thymus/ leukocytes
XI A	12.4	6	green rice shoots
XI B	12.9	6	green rice shoots
XII	19	7	human/ murine
XIII	< 10	0	parovirus
XIV	13-19	2	symbiotic fungal/ bacteria

4.3.2 GIVA $cPLA_2$

The cytosolic phospholipase A_2 (GIVA PLA_2 or $cPLA_2\alpha$) was first reported in human neutrophils and platelets, by Leslie and Kramer in 1986.[24] Now six members of the $cPLA_2$ group IV are known.

[24] {Dennis 2011 #8}

Table 2: Dennis classification of cytosolic phospholipases A_2

group	source	molecular weight [kDa]	features	alternate name
IV A	human/ murine	85	C2 domain	$cPLA_2\alpha$
IV B	human	114	C2 domain	$cPLA_2\beta$
IV C	human	61	acylated	$cPLA_2\gamma$
IV D	human/ murine	92-93	C2 domain	$cPLA_2\delta$
IV E	murine	100	C2 domain	$cPLA_2\varepsilon$
IV F	murine	96	C2 domain	$cPLA_2\xi$

The GIVA $cPLA_2$ is a 85-kDa protein and contains of a N-terminal Ca_2^+ binding domain (C2 domain) and a C-terminal catalytic domain, linked by a flexible tether.[25] The binding of the GIVA PLA_2 to the membrane is mediated through three mechanisms: Ca^{2+}-mediated translocation, binding of secondary lipid messengers, and phosphorylation.[26] The GIVA $cPLA_2$ is regulated by intracellular calcium concentration. Calcium binding to the C2 domain leads to a translocation of the enzyme from the cytosol to the phospholipid membrane. Phosphorylation of the enzyme regulates the activity at the phospholipid membrane and its cellular functions. The level of phosphorylation, especially the level of Ser-505 phosphorylation by mitogen-activated protein kinases (MAP kinase), has been implicated in the activation of GIVA PLA_2 in response to various cellular stimuli.[27]

Once GIVA PLA_2 has localized to the membrane, the active site is in the correct orientation to allow substrate molecules to enter the active site. The C-terminal domain of GIVA PLA_2 alone does not have phospholipase activity.[28] Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) are described substrates of GIVA PLA_2. It is also known that $cPLA_2$ can be activated in a Ca^{2+} independent manner, by binding to the lipid second messenger, phosphatidylinositol-4,5-bisphosphate (PIP_2). Furthermore, it has been demonstrated that the presence of PIP_2 causes a 100-fold increase in GIVA $cPLA_2$ activity.[29]

[25] {Cao 2013 #6}
[26] {Balsinde 2002 #3}
[27] {Dennis 2011 #8}
[28] {Dennis 2011 #8}
[29] {Lucas 2005 #26}

The cytosolic phospholipase A_2 is ubiquitously present in all tissues. It shows a stringent fatty acid preference for the sn2-position. The primary function of the enzyme is the release of AA. It is therefore involved in inflammatory processes.[30]

The enzyme has two phosphorylation sites (serine 505, serine 727) for mitogen-activated protein kinase and MAPK-activated protein kinase (MAPKAPK), whose phosphorylation results in activation of the enzyme (in the presence of calcium). During apoptosis $cPLA_2\alpha$ is cleaved by caspase 3 which leads to inactivation of the enzyme.[31,32]

4.3.3 Calcium-independent phospholipase A_2

The Ca^{2+}-independent phospholipase A_2 was first purified from $P388D_1$ macrophage-like cell line in 1994. This type of superfamily is characterized by its calcium-independent phospholipase activity and is ubiquitous in various tissues. The name in general refers only to the GVI PLA_2, the first member of the group.[33,34] To date, the GVI PLA_2 (now GVIA) includes six different members namely GVIA-1 ($iPLA_2$), GVIA-2 ($iPLA_2\beta$), GVIB ($iPLA_2\gamma$), GVIC ($iPLA_2\delta$), GVID($iPLA_2\varepsilon$), GVIE ($iPLA_2\zeta$), and GVIF ($iPLA_2\eta$). All of the members have different physiological functions and localize to different organelles but share the same catalytic domain, a consensus lipase motif with a serine residue at the active centre (GXSXG). Recent studies have shown that the $iPLA_2$ enzymes play a major role in inflammation as it provides arachidonic acid and cell proliferation of lymphocytes. Although $iPLA_2$s are associated with diseases such as diabetes mellitus, Barth syndrome and neurological disorders like Parkinson's disease and neuroaxonal dystrophy.[35] Due to that fact, several studies focus on the research on GVIA $iPLA_2$ inhibitors like Bromoenol lactone (BEL) which is the most important irreversible inhibitor of the enzyme.[36]

4.4 Phospholipase D

The Phospholipase D (PLD) is a phosphatidylcholine-hydrolyzing enzyme that functions in multiple cellular pathways and is found in diverse organisms from bacteria to human. These enzymes are well known to generate the lipid second messenger phosphatidic acid (PA) and a free headgroup by employing water as a nucleophile to hydrolyse phospholipid substrates. PA is also a precursor to other lipid signaling molecules and can be converted into diacylglycerol (DAG) by lipid phosphatase and lysophosphatidic acid (lyso-

[30] {Balsinde 2002 #3}
[31] {Krönke 2002 #19}
[32] {Taketo 2002 #45}
[33] {Winstead 2000 #49}
[34] {Dennis 2011 #8}
[35] {Dennis 2011 #8}
[36] {Farooqui 2006 #11}

PA) by phospholipase A_2. [37] PA is a critical lipid second messenger for a range of signaling cascades, but makes up 1-4 % total lipid in the cell.[38]

The two classic mammalian isoforms of PLD are PLD1 and PLD2. PLD activity in mammalian cells is low and is transiently stimulated by protein kinases and guanosine-5'-triphosphate (GTP) binding proteins of the adenosine diphosphate (ADP)-ribosylation and Rho families. Mammalian phospholipases D are also potently stimulated by the co-factor phosphatidylinositol 4,5-bisphosphate.[39] The two PLD isoforms may have different localization and play different roles in cells but are expressed in nearly all mammalian tissues. PLD activity has been first described in plants in the early 1980s, to date more than 4000 PLD enzymes have been entered in National Center for Biotechnology Information (NCBI) GenBank.[40] Because of the fact that phospholipase D1 and D2 play roles in pulmonary embolism stroke, hypertension, neurite outgrowth, Parkinson's and Alzheimer's disease, an extensive effort in the development of drug-like, isoform-selective PLD inhibitors exists.[41]

4.4.1 PLD activation

As mentioned before phosphatidic acid acts as a lipid second messenger, which is located at the intersection of several lipid metabolism and cell signalling events including membrane trafficking, survival, and proliferation. For generating this second messenger phospholipase D enzymes are required. They share a highly conserved "HKD" domain necessary for catalysis. PLD regulation in cells falls into two major signalling categories. It needs to be activated by a variety of both receptor-tyrosine kinase (RTK) and GPCR stimulus.[42]

[37] {Peng 2012 #31}
[38] {Dennis 2010 #9}
[39] {Peng 2012 #31}
[40] {Selvy 2011 #41}
[41] {Peng 2012 #31}
[42] {Scott 2013 #40}

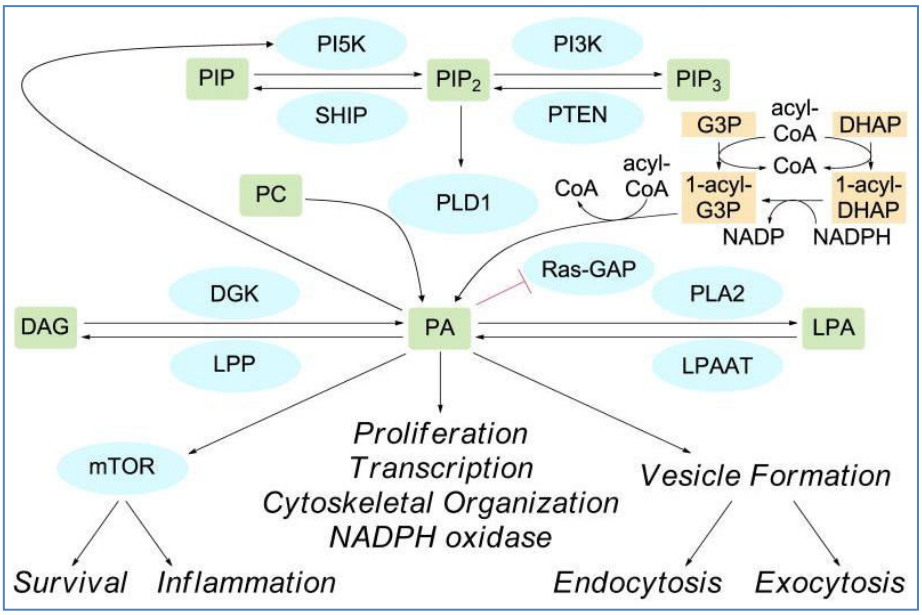

Figure 3: Phospholipase D and phosphatidic acid signalling according to the work of Selvy et al, 2011: PA can be generated de novo or in response to cell signalling pathways. Signal generated PA is formed by enzymes that modify existing lipids[43]

For the activation of several physiological functions in the cell a GPCR is necessary. The downstream signalling targets (like PLD) of these cell surface receptors are key enzymes for drug development. The expression levels of the PLD1 and PLD2 gene are under developmental control and can also show a discrepancy during cell differentiation. A binding of the GPCR agonist leads to a dissociation of the trimeric G-protein into GTP-α_q and Gβγ, which then leads to PLD activation, resulting in the release of PA. PA can then be hydrolyzed to diacylglycerol by a lipid phosphate phosphatase (LPP). (Illustrated on figure 3)

[43] {Selvy 2011 #41}

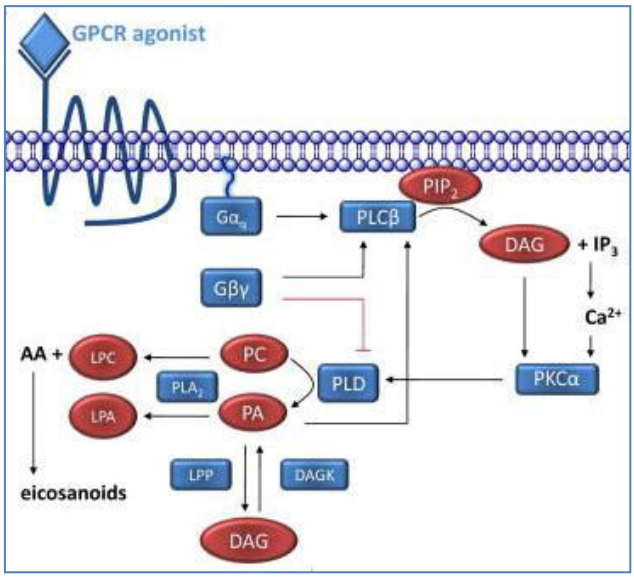

Figure 4: PLD activation by dissociation of Gα and Gβγ heterotrimeric G proteins upon agonist stimulation[44]

4.4.2 Phospholipase D1

The two isoforms of mammalian PLDs share about 50 % amino acid similarity. PLD1 is a 120 kDa protein which was first identified in yeast *Saccharomyces cerevisiae.* It has a low basal activity and responds to protein kinase C (PKC) and to members of the Rho and Arf families of small G proteins.[45] PLD1 is primarily localized in vesicular structures such as endosomes, lysosomes and autophagosomes. PLD1 occurs in two splice variants named PLD1a and PLD1b.

All members of the PLD superfamily own two highly conserved phosphatidyltransferase HKD catalytic domains (HKD1 and HKD2), which are vital to the lipase activity. Upon stimulation, the PLD1 translocates to the intracellular membranes, thus it is palmitoylated on two cysteine residues in the PH domain which serves as anchorage point. On the N-terminus PLD1 as well as PLD2 harbour lipid binding Pleckstrin homology domain (PH) and pbox consensus sequence (PX) domains. The PLD1 activity can be significantly increased by phosphatidylinositol 4,5-bisphosphateand otherwise decreased by the addition of sodium oleate.[46] Studies confirmed that in vivo but not in vitro, PLD activity is also modulated by changes in Ca^{2+} concentration.[47,48]

[44] {Selvy 2011 #41}
[45] {Hu 2003 #14}
[46] {Brandenburg 2014 #4}
[47] {Hu 2003 #14}
[48] {Selvy 2011 #41}

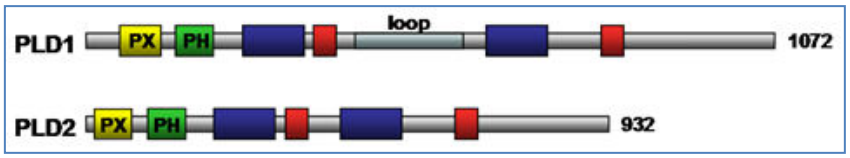

Figure 5: Schematic structure of mammalian phospholipase D enzymes: PX (yellow)and PH domain (green), phospholipase D-specific catalytic domains (blue), HKD domain (red), and loop (light blue). Amino acid residues: PLD1-1072, PLD2-932[49]

4.4.3 Phospholipase D2

Phospholipase D2 is a 105 kDa protein which is localized on chromosome 17. As well as PLD1, PLD2 requires a co-factor for activation, but lacks the 116 amino acid loop region following the first HKD motif. The activity of PLD2 is positively regulated by phosphatidylinositol 4,5-bisphosphate and Ral, and negatively regulated by cytoskeletal proteins. In contrast to PLD1, PLD2 exhibits high basal activity that is apparently controlled by repression.[50]To date, α- Synuclein and β-synuclein were identified as cytosolic proteins that have the capacity to inhibit PLD2 activity in vitro. Some other proteins that may decrease PLD2 activity are PIP_2 -binding proteins such as fodrin.[51]

PLD2 primarily localized to the plasma membrane, in contrast to PLD1 which is localized solely to peri-nuclear regions. PLD2 can be translocated into the submembranous vesicles by serum and ruffling membranes by epidermal growth factor (EGF).[52] Many reports have concluded that PLD2 is critically linked to the signals that mediate the processes involved in tumorigenesis and metastasis. Additionally, studies confirm that PLD1 as well as PLD2 became implicated with other pathological disorders, such as Alzheimer's disease and hypertension. These observations suggest that the PLDs could be a potential therapeutic target for any of these diseases.[53]

4.4.4 Phospholipase D1, D2 and $cPLA_2$ in health and disease

The enzymatic processing of phospholipids, which are major components of cell membranes, converts these molecules into lipid mediators or second messengers. These are known to regulate physiological and pathophysiological functions.

[49] {MichelleBamji-Mirza, Zemin Yao 2011 #27}
[50] {Steed 1998 #42}
[51] {Liscovitch 2000 #25}
[52] {Colley 1997 #7}
[53] {Lee 2013 #20}

Table 3: Summary of cPLA2, PLD1 and PLD2 roles in health and disease according to Paramjit et al

Involved phospholipase	Disease	Functional role
$cPLA_2$-IVA	Parkinson's disease	Involved in MPTP-induced dopamine depletion
	Asthma	Contribute to development of asthma
	Arthritis	Contributes collagen-induced arthritis
	Intestinal polyposis	Regulates expansion of polyps
	Colorectal cancer	Regulates pro apoptosis signal
	Platelets dysfunction	Regulates production of TXA2 and 12-hydroxyeicosatetraenoic acid, and platelet aggregation
PLD1	Brain ischemia	Protects neuronal cells from apoptotic condition
	Alzheimer`s disease	Up-regulated expression and activity of PLD
	Bleeding disorder	Regulates integrin αIIbβ3 activation and aggregate formation
	Breast cancer	Up-regulated PLD1 may contribute to tumorgenesis Over-expressed PLD1 associates with poor prognosis
	Melanoma, lung carcinoma, breast cancer	Promotes tumor growth and metastasis in the tumor environment

PLD2	Colorectal cancer	Polymorphism of PLD2 is associated with colorectal cancer Up-regulated PLD2 may contribute to tumor size and survival
	Brain ischemia	Protects neurons from ischemia
	Alzheimer`s disease	Deletion of PLD2 rescues deficits SwAPP mouse
	Renal cancer	Up-regulated PLD2 may contribute to tumorgenesis

PLD1 and PLD2 are both expressed in the brain during development and postnatal life. PLD1 are highly expressed in oligodendrocytes, however PLD2 is expressed in astrocytes and both in ependymal cells. In summary, both types of PLDs regulate various neuronal activities and have been implicated in survival of neuronal cells in ischemia. The relationship between PLD1 and PLD2 and Alzheimer`s disease (AD) has been first described in 1986.[54] Kanfer et al measured a decrease in PLD activity in AD brain homogenates relative to those from control patients. Cytosolic PLA_2 expression levels are lower in the brain, in contrast to those of PLD but have also crucial function in the brain.[55] The $cPLA_2$ released AA and docosahexaenoic acids are known to regulate the release, uptake and transport of neurotransmitters. Measured levels of $cPLA_2$ in occipital cortex and cerebellum of Alzheimer's patients were elevated above those in normal persons.[56]

Immunohistochemical studies suggested that PLD1 as well as PLD2 were randomly localised in resting platelets, but became rapidly translocated to the plasma membrane due to stimulation via thrombin. Those results confirmed that there is a role for PLD in platelet activation.[57] Additionally, PLD1 is involved in secretion of von Willebrand factor (vWf), which is a major clotting factor in the coagulation cascade of blood. The deficiency of this factor results in the most common inherited bleeding disorder namely von Willebrand disease. A knockdown of PLD1 showed a dramatically decrease in secretion of vWF.[58] Cytosolic phospholipase A_2 catalyzes the release of AA from

[54] {Kanfer 1986 #17}
[55] {Oliveira 2010 #30}
[56] {Stephenson 1996 #43}
[57] {Vorland 2008 #48}
[58] {Tappia #46}

glycerophospholipids, leading to thromboxane A2 production.[59] Studies suggested that the mutation in *PLA2G4A* gene is associated with platelet dysfunction in human. Patients with heterozygous mutation in this gene showed a reduced thromboxane production. Furthermore platelet aggregation and degranulation, which is induced by adenosine diphosphate or collagen, were diminished.[60]

4.5 Arachidonic acid and prostaglandins

As previously described, AA is an essential fatty acid and a precursor for all prostaglandins, thromboxanes, and leukotrienes. Virtually all cellular AA is esterified in membrane phospholipids where its presence is tightly regulated through multiple interconnected pathways. GIVA PLA_2 is considered as a central enzyme for mediating eicosanoid production and plays a major role in inflammatory diseases. Patients with a deficiency of the enzyme, and also knockout mouse models have shown decreased eicosanoid production and an easing in the effects of inflammatory diseases.[61] Studies confirmed that AA release is also mediated by PLD and phosphatidic acid phosphohydrolase in human keratinocytes.[62] The cyclooxygenase enzymes (COX-1, COX-2) are the key enzymes in the next step of converting AA into prostaglandins. Prostaglandin E2, a key product of COX-2, is known to have an immunomodulatory role.[63]

4.5.1 Eicosanoid biosynthesis and Prostaglandin E2

Eicosanoids are a family of oxygenated metabolites of AA (C_{20} fatty acid), including the prostaglandins, thromboxanes, leukotrienes and lipoxin. All mammalian cells except erythrocytes synthesize eicosanoids. They are lipid mediators derived from phospholipase-released AA that are involved in numerous homeostatic biological functions as well as in a number of disease states as inflammation.[64]

Three enzymatic pathways are responsible for their synthesis in mammalian cells: lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome P450 (CYP). All eicosanoids function locally at the site of synthesis, through receptor-mediated G-protein linked signaling pathways. A key enzyme in this pathway is cyclooxygenase, whose inducible isozyme COX-2 is upregulated in inflammatory diseases and is traditionally regarded as one of the earliest biomarkers of the inflammatory cascade in arthritis.[65] COX peroxidase activity catalyzes the generation of the unstable intermediate, prostaglandin H_2, from arachidonic acid and oxygen. A specific prostaglandin synthase, PGES,

[59] {Wong 2002 #50}
[60] {Adler 2008 #1}
[61] {Dennis 2011 #8}
[62] {Lefkowitz 2002 #21}
[63] {El-Rifaie 2015 #10}
[64] {O'Donnell 2009 #29}
[65] {Sano 1992 #37}

converts PGH_2 to PGE_2, the most abundant pro-inflammatory PG associated with inflammatory conditions. The rate of PGE_2 synthesis and the resulting inflammatory process can be affected by additional factors, such as local availability of AA. Due to that, in most physiological conditions the rate of PGE_2 synthesis is controlled by local expression and activity of COX2. PGE_2 (molecular mass of 352 Da), was first recognized as a biologically active factor in the 1960s and until know it has been shown to regulate multiple aspects of inflammation and multiple functions of different immune cells.[66] Secreted PGE_2 acts in an autocrine or paracrine manner through its four cognate G protein-coupled receptors EP1 to EP4. Under physiological conditions, PGE2 acts in biological functions, such as regulation of immune responses, blood pressure, gastrointestinal integrity, and fertility.[67] Due to the fact that PGE_2 is involved in all signs of classic inflammation such as pain, redness and swelling it is from particular interest.[68] Moreover, it coordinates the whole process in both pro-inflammatory and anti-inflammatory directions.

4.6 The role of Endothelin

Endothelin is a 21 amino acid peptide with two sets of intrachain disulfide linkages, which is produced by endothelial cells. It was first extracted from the supernatant of cultured aortic porcine cells in 1988 by Yanagisawa et al. Endothelins occur in three isoforms, namely endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). The three forms differ slightly in their amino acid sequence. Depending on the receptor interaction, endothelin causes vasoconstriction (ETa receptor) or vasodilatation (ETb receptor). ET-1 is the major player in terms of vasoregulation and is mainly produced by endothelial cells and to a lesser extent by vascular smooth muscle and perivascular nerve cells. ETa receptors occur most frequently on vascular smooth muscle cells and are mainly responsible for endothelin-induced constrictions. ETb receptors are expressed in vascular beds primarily on endothelial cells, where it dilates the vascular muscles cause an activation of NO synthesis.[69,70] In addition to the affinity for the ET isoforms, the main differences between ETa and ETb receptor is their differential expression and their second messenger cascades.

[66] {Kalinski 2012 #16}
[67] {Legler 2010 #22}
[68] {Funk 2001 #12}
[69] {Hirata 1993 #13}
[70] {Tsukahara 1994 #47}

5 Aim of the thesis

As mentioned in the introduction, PLD1, PLD2 and $cPLA_2$ have been suggested to play a role in mobilization of arachidonic acid and stimulation of prostaglandin synthesis, in numerous cell types. The aim of the thesis was to use small interfering RNA duplexes (siRNA) to silence one of those PLAs, to examine which of the three phospholipases is directly responsible for arachidonic acid liberation and prostaglandin E_2 formation. The latter is to be identified by means of negative chemical ionisation, the mass spectrum and the retention time. Quantification took place by using D_4-PGE_2 as an internal standard. The success of silencing was examined by Western Blot and PCR.

6 Materials and methods

6.1 Cell culture

The mouse calvaria-derived pre-osteoblastic cell line MC3T3-E1, which was used for the experiments, has been established from C57BL/6 mouse calvaria. They differentiate from preosteoblasts to mature osteoblasts as a function of time in culture.[71] Before starting the procedure, a cryogenic vial containing 1 ml cell solution was taken out of the liquid nitrogen tank and thawed in warm water. The cells were then transferred to a 30 ml tube and resuspended in 9 ml minimum essential medium (MEM) (*GIBCO Invitrogen, USA)* containing 5% fetal calf serum (FCS) *(PAA, Austria).* To eliminate all dimethyl sulfoxide residues, cells were centrifuged and the supernatant was removed. After resuspending in 10 ml medium, cells were transferred into a culture flask and incubated at 37°C with 5% carbon dioxide (CO_2). The cells were cultured for two weeks.

6.1.1 Cell blotting

After heating the reagents in a 37°C water bath, the culture medium of subconfluent MC3T3-E1 cell cultures was removed and the cells were washed twice with phosphate-buffered saline (PBS)(*LKH Graz, pH 7.2*). 2 ml trypsin solution (*Promega*) was applied and incubation took place for 2.5 to 3 minutes on a 37°C plate to displace the cells from the flask surface. The reaction was stopped by adding 8 ml MEM. After resuspending, the cells were centrifuged and the supernatant was removed. The cell pellet was resuspended in 10 ml medium and 10 µl of the cell suspension were taken out in a counting chamber. After determination of the cell number, cells were adjusted to 80 000 cells/2 ml. The 2 ml aliquots were transferred to 6-well culture plates and stored for 24 hours at 37°C with 5% CO_2.

[71] {Sudo 1983 #42}

6.1.2 Transfection

Table 4: Used siRNAs

siRNA	Description	Company	Cat. nr.	Tube ID	Concentration
Negative control		Qiagen	1022076		5 nM
PLA2g4a_5(cPLA2)	**GS18783**	Qiagen	SI02668330	2155849	1 nM
PLA2g4a_6(cPLA2)	**GS18783**	Qiagen	SI02688091	2155850	1 nM
PLA2g4a_7(cPLA2)	**GS18783**	Qiagen	SI02710015	2155851	1 nM
PLA2g4a_8(cPLA2)	**GS18783**	Qiagen	SI02734312	2155852	1 nM
Mm_Pld1_5	**GS18805**	Qiagen	SI02734326	2173138	1 nM
Mm_Pld1_6	**GS18805**	Qiagen	SI04920349	2173139	1 nM
Mm_Pld1_7	**GS18805**	Qiagen	SI04920356	2173140	1 nM
Mm_Pld1_8	**GS18805**	Qiagen	SI04920363	2173141	1 nM
PC-PLD2 siRNA ®		Santa Cruz	sc-270132		10 µM

Before starting the transfection, MEM was heated in a 37°C water bath and solution B containing 21 µl MEM and 4 µl oligofectamine transfection reagent *(Gibco)* was prepared and incubated for 10 minutes at room temperature (RT). Per experiment two controls, one and each in duplicate were used. Each batch was carried out in triplet. For both, siRNAs as well as the siRNA-negative controls, one 1.5 ml Eppendorf tube containing 165 µl MEM was prepared. 10 µl of the siRNAs and the control were pipetted in the appropriate tubes and 25 µl of solution B were added. During an incubation time of 20 minutes at RT, the supernatant of the cells was removed and cells were washed by adding 1 ml MEM. After removing the medium transfection started by adding 800 µl MEM in each well and 200 µl of the corresponding transfection mix. Transfection took place for 4 hours at 37°C with 5% CO_2. To gain the final concentration of 100 nM/2ml for siRNAs and the control, 1 ml of the post transfection medium (20% MEM +5% FCS) were added. Cells were incubated at 37°C with 5% CO_2 for 2 days without changing the medium.

6.1.3 ET-1 stimulation

Two days after the transfection with siRNAs, the PG E2-B1 buffer containing 60 ml MEM, 0.2 % FCS and 1.8 mM Ca^{2+} was prepared and stored in the 37°C water bath. This buffer was then used to prepare the ET-1-buffer with a final endothelin *(Sigma Aldrich)* concentration of 50 nM in 2 ml wells.

At first, the medium was removed and exchanged for 1.7 ml of the PG E2-B1 buffer and cells were incubated for 15 minutes at RT. siRNA transfected cells and one of the siRNA-controls were then stimulated with 200 µl ET-1-buffer. The other control was treated with 200 µl PG E2-B1 buffer.

Stimulation took place for 30 minutes at 37°C without CO_2. To stop the reaction, the supernatant was transferred into refrigerated polypropene vials (PP) *(La Fontaine)* containing 1 ml 0.5% and 1 ml 0.02% formic acid, to stabilize the contained prostaglandins. 50 µl D_4-PGE_2 *(Cayman)* was added to each vial, as an internal standard for the quantification of PGE_2.

6.1.4 Cell harvest

For Western Blot analysis the cellular processes were suppressed by washing the cells twice with 5 ml cold PBS (stored on ice). For PCR, the cells were washed conclusively with 5 ml cold PBS containing EDTA *(Sigma Aldrich)*. Cell lysis was initiated by addition of either 100 µl of lysis buffer (pH 7.4, 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 2 nM Na-orthovanadate, 10 mM NaF, 1% (v:v) Triton X-100,10% (v:v) glycerol, protease inhibitor cocktail tablets, pH 7.4) for Western Blot or 600 µl RLT-buffer *(Quiagen)* for PCR analysis. The cells treated with RLT-buffer were immediately scrapped using a rubber policeman and transferred into 1.5 ml Eppendorf tubes. The dishes with the lysis buffer cells were shaken for 15 minutes at 4°C. Subsequently 135 µl of the cell lysate were transferred into 1.5 ml tubes and remaining cell debris was removed by centrifugation at 14.000 *g* for 10 minutes at 4°C. 30 µl of the supernatant was then transferred into new tubes and stored at -20°C over night as well as the RLT-cell lysate.

6.2 Prostaglandin extraction

After stabilization of the prostaglandins, the extraction followed by adding 4.5 ml ethylacetate *(Merck, Germany)*. Samples were mixed for 10 min in an overhead shaker and then centrifuged for 3 minutes with 3500 rpm. The supernatant was transferred into 5 ml glass vials and dried completely at 39°C under a nitrogen stream.

For the purposes of PG quantification by gas chromatography/negative ion chemical ionization (GC-MS/NICI), PFBBr (Pentaflourobenzyl bromide) (*ABCR)* ester derivatives of the monocarboxylic forms of PG were prepared. The reaction was carried out by incubating the final lipid extract in the presence of the catalyst DIPEA (N,N-Diisopropylethylamine) (*Sigma-Aldrich*).

Therefore samples were derivatized with 50 µl PFBBr and 10 µl DIPEA. After vortexing, the samples were incubated at room temperature for 10 minutes, and dried under nitrogen stream at 39°C. Furthermore 50 µl methoxamine (MOX) *(Pierce)* were added to prevent the formation of multiple derivatives when enols are present during silylation. After vortexing, samples incubated for two hours at 75°C.

For the MOX-extraction 0.4 ml aqua bidest *(Fresenius, Austria)* and 2.5 ml n-Hexane *(Merck)* were added. Samples were mixed for 10 min in an overhead shaker and then centrifuged for 3 minutes

with 3500 rpm. The supernatant was transferred into 5 ml glass vials and dried completely at 39°C under a nitrogen stream. Furthermore the samples were dissolved in 50 µl MSTFA (N-Methyl-N-trimethylsilyltrifluoroacetamide) (*ABCR*) containing 1% trimethylchlorosilane (TMCS) silylation reagent. TMCS aids derivatization of early eluting compounds or amides, secondary amines, and hindered hydroxyls not derivatized by MSTFA alone. Samples were incubated for 20 minutes at RT and dried under nitrogen stream at 39°C. For the last step in derivatization, dried samples were dissolved in 50 µl n-Hexane containing 1% N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and transferred into microvials for the gas chromatographic and mass spectrometric (GC-MS) measurement.

6.3 PGE_2 measurement

The contained prostaglandins were measured by using gas chromatography and mass spectrometry (*Thermo Scientific ISQ*). 1 µl of each sample was injected (10 µL 700 series hand fitted MICROLITER syringe, *Hamilton*, Switzerland) splitless into the analytical column (ID-BPX5-0.25, 15 m x 0.25 mm). Methane was used as chemical ionization gas at a constant flow of 1.5 ml/min. The temperature parameters were set as follows: injector at 260°C, GC/MS interface at 310°C. Oven program: the initial temperature was set at 160°C for 1 minute, 310°C for 4 minutes. Helium was used as carrier gas. Run timeout was set at 10 minutes. The samples were ionized with methane by negative ion chemical ionization (NICI). NICI was accomplished with electron energy of 70 eV.

6.4 Protein quantification with BCA Protein Assay

The determination of the total protein concentration was essential for Western Blot analysis. Therefore the Micro BCA™ Protein Assay Kit *(Pierce)* was used. For the preparation of diluted Albumin (Bovine serum albumin-BSA) standards 20 mg BSA *(Sigma)* were dissolved in 10 ml aqua bidest. From the prepared calibration series with the concentrations 20, 15, 10, 7.5, 5, 2.5 and 1.25 µg, 10 µl of each were pipetted in a 96-well microplate (all measurements were performed in duplicate). The same amount was taken from each sample. 50 parts of reagent A and 1 part reagent B were mixed to prepare Micro BCA™ working reagent. 200 µl of the working reagent were pipetted into each well. The microplate was incubated for 90 minutes, protected from any light. Finally the total protein concentration was measured at a wavelength of 540 nm according to the manufacturer's instructions, by using a plate reader *(Bio Rad)*. A protein concentration of 35 µg was used for the subsequent Western Blot.

6.5 Western Blot analysis

After the measurement protein measurement, lysates (30 µl) were diluted in 10 µl NuPAGE lithium dodecyl sulphate (LDS) sample buffer and 3 µl NuPAGE sample reducing agent to a final volume of 43

µl. Furthermore, all samples were shaken boiled for 10 minutes at 70°C. After washing the slots of the ready prepared 4-12% NuPageBis-TRIS-Gel 1.5 mm x 10 well *(Invitrogen)* with NuPage 2-(*N*-morpholino)ethanesulfonic acid (MES)- sodium dodecyl sulphate (SDS)-Running buffer 20x *(Invitrogen)*, the first and the last slot were loaded with 15 µl *Novex* LC1001 standard. All other slots were used for the samples. After refilling the chamber with the running buffer, the electrophoresis started with 130 V for 8o minutes. Immediately after running the electrophoresis, proteins were transferred to nitrocellulose membranes (*Invitrogen*, LC 2001) by using a transfer puffer (100 ml 10x running buffer + 200 ml methanol in 1l aqua bidest). Following transfer conditions were used: 0.3 Ampere for 2 hours.

After blocking the membrane with blocking buffer (5% non-fat milk powder in TBS-T) for 30 minutes at RT and washing with 1x Tris-buffered saline (TBS-T), the blots were incubated with the corresponding primary antibody (AB) (diluted in 10 ml 5% BSA without IgG/TBS-T pH 7.4). Followed by the addition of 0.2% gentamicin *(Gibco)*, incubation started for 2 h at room temperature, depending on the antibody respectively. The membranes were washed 3 x 10 min in TBS-T buffer, and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h in 5% non-fat milk powder/TBS-Tat room temperature. After another 3 x 10 min washing with TBS-T, the blot was incubated in chemiluminescent substrate *(Millipore)* for 5 minutes and signals were detected by exposure to a photographic film. β-actin was used as loading control. Therefore the membrane was stripped for 7 minutes right after exposure and re-probed with anti-mouse IgG secondary antibody.

Table 5: List of antibodies, all obtained from Santa Cruz Biotechnology

AB Name	Product Nr.	Size	Secondary AB
ß-Actin	sc-47778	44 kDa	anti-mouse IgG
$cPLA_2$(H-12)	sc-376636	85-114 kDa	anti-mouse IgG
PC-PLD2 (V20)	sc-48270	117 kDa	anti-goat-IgG
PC-PLD1 (C-17)	sc-17848	115-120 kDa	anti-goat-IgG

6.6 RNA reprocessing and quantification with Qubit

The cellular RNA from osteoblast like MC3T3-E1 cells was isolated using ready prepared RLT as lysis buffer. Before starting reverse transcriptase PCR the concentration of the RNA of the samples needed to be measured. RNeasy Mini Kit *(Quiagen)* was used to purify the small amounts of RNA. First the samples were vortexed and subsequently transferred into Quiashredder tubes. After 2 minutes full speed centrifugation (13 000 rpm), 550 µl ethanol were added to the lysate, to create conditions that promote a selective binding of RNA to the membrane in the tubes. The samples were carefully mixed with the pipette tips and 550 µl were converted in a spin column and centrifuged for

15 seconds with 10 000 rpm. The eluate was aspirated and the rest of the RNA lysate was transferred and centrifuged under same conditions. These steps were necessary to efficiently wash away contaminants. To wash the spin column, 700 µl RW1 buffer were added, followed by another centrifugation step for 15 seconds at 10 000 rpm. Eluate and tubes were discarded and 500 µl RPE buffer were added (a new 2 ml tube was used). The same step was repeated once more, eluate and tubes were discarded and the spin column was transferred in a new 1.5 ml collection tube. After adding 100 µl RNase free water and 1 minute centrifugation with 10 000 rpm, the high-quality RNA was eluted. To prevent contaminations and protect the purified RNA from degradation 2 µl RNasin ribonuclease inhibitor *(Promega)* per sample were added.

After purification of RNA the Qubit® fluorometer was used for the measuring of the RNA concentration. Two standards were used for the instrument calibration. Before running the procedure, the working solution was prepared: 1 µl Reagent and 199 µl ready prepared buffer. By addition of 2 µl sample the reaction started with 2 minutes incubation at RT. Immediately afterwards, the samples were measured and the RNA concentration per sample was calculated.

To digest single-and double-stranded DNA to oligodeoxy-ribonucleotides containing a 5'- phosphate, samples were treated with DNase *(Invitrogen)* in the following order: Diethylpyrocarbonate (DEPC)-treated water to a volume of 20 µl, 2 µl 10x DNase I Reaction Buffer, 2 µg RNA sample and 2 µl DNase I, Amplification Grade, 1 U/µl. After an incubation time for 15 minutes at RT, and stopping the reaction by adding 2 µl ethylenediaminetetraacetic acid (EDTA), samples were boiled for 10 minutes at 65°C. Conclusively each sample was diluted with 74 µl RNase free water at a final concentration of 100 ng/ 5 µl.

6.7 Reverse-transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed with QIAGEN®OneStep RT-PCR Kit. According to the manual's instruction the kit includes optimized components that allow both reverse transcription and PCR amplification to take place in the same reaction mix in a "one-step" reaction. For running the PCR, components were prepared as followed: 100 ng template RNA, 400 µM from each dNTP, 10 µl 5x QIAGEN OneStep RT-PCR Buffer, gene-specific primers (for $cPLA_2$, PLD2 or PLD1) in a final concentration of 0.6 µM and 2 µl of QIAGEN OneStep RT-PCR Enzyme Mix (containing Reverse Transcriptase and HotStarTaq DNA Polymerase). The kit contained also aqua bidest to obtain a final volume of 50 µl.

Conditions for the polymerase chain reaction were selected as following: reverse transcription reaction was conducted for 30 minutes at 50°C, followed by initial PCR activation step for 15 minutes at 95°C, 35 amplification cycles each and performed for 30 seconds at 94°C, followed by annealing for 30 seconds at 59°C, elongation was set for 1 minute at 72°C and a final elongation step for 10

minutes at 72°. RT-PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed for each experiment, to ensure equal RNA loading of the samples.

Table 6: List of primers used for quantitative reverse transcriptase PCR obtained from Quiagen (mPLD2 from Santa Cruz)

Name ofthe primer	Internal Code	Product Nr.	Sequence
mPLA2G4A 570-F	A2F	1411812	5'- GGCACAGCTACATTCCCTGT -3'
mPLA2G4A 570-R	A2R	1411813	5'- TAAAGGTGACAGGCTGGC -3'
mPLA2G4A 867-F	A1F	1411810	5'- GTGTCTGGGGCAGTGCCTTT -3'
mPLA2G4A 867-R	A1R	1411811	5'- GTTGAAAATGGCGATTCGGG -3'
mPLD1_v12_119-F	D1 F	1411794	5'-AGTGCTTCAGACTTGTCCTGGGTT-3'
mPLD1_v12_119-R	D1 R	1411795	5'-TATGGTAGCGTTTCGAGCTGCTGT-3'
mPLD2_309-F	D6 F	1411804	5'-GTGCCACTGTGCAGGTCTTGAGG-3'
mPLD2_309-R	D6 R	1411805	5'-GCAGAATAGCCTGGATGGAG-3'
mGAPDH-F		1414441	
mGAPDH-R		1414440	

For the electrophoresis ReadyAgarose Gel from *Bio Rad,* containing 20 wells and a load volume of 20 µl and supplemented with 0.1% ethidium bromide was used. 15 µl of each sample and 5 µl standard was prepared with 5 µl loading dye. The run was carried out with 1 x TBE buffer at 100 V constantly for 60 minutes.

7 Results

7.1 $cPLA_2$ silencing experiments

The first testing was used to see whether the transfection with different siRNA duplexes is specific for $cPLA_2$ and the resulting silencing worked out and is reproducible, to see if these changes have an effect on arachidonate liberation and prostaglandin E_2 formation. siRNA functions by causing mRNA to be broken down after transcription resulting in no translation of the gene. This effect was tried to visualize by using RT- PCR at DNA level and Western Blot for proteins. After stabilization of prostaglandins and extraction, they were measured by using gas chromatography and mass spectrometry.

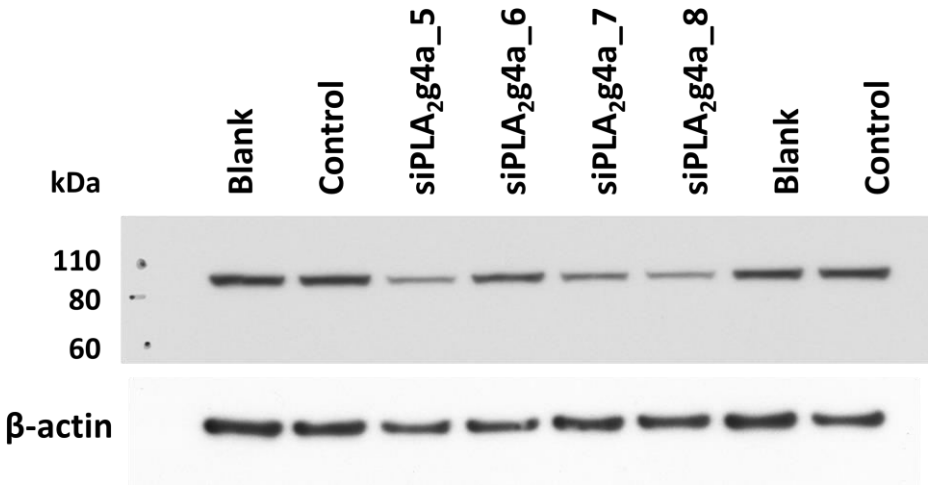

Figure 6: Western Blot analysis of $cPLA_2$ in MC3T3 cells (subculture 21) after transfection with different siRNAs and stimulation with ET-1

30 µl MC3T3 cell lysate was loaded onto the gel from untreated cells (blank) and after transfection with a final concentration of 100 nm PLA2g4a_5-8 ($cPLA_2$) and stimulation with 50 nm ET-1 for 30 minutes. Control was treated with ET-1 without any transfection, respectively. Subsequent treatment of the membrane with $cPLA_2$ primary antibody revealed a band at approximately 100 kDa. β-actin was used as loading control.

7.1.1 Western Blot analysis of $cPLA_2$

Cell lysate for Western Blot analysis was collected from untreated cells and after treatment with siRNA duplexes. In figure 6 the Western Blot of MC3T3-E1 cells using $cPLA_2$ antibody sc-376636 (H12) *(Santa Cruz)* is shown. Compared with the expected bands at 85-114 kDa, the blot shows all bands with apparent molecular weights at approximately 95 kDa. There are visible differences in the band intensities between treated and non-treated control. The results for $cPLA_2$ confirmed that the

silencing on protein level was successful. Unfortunately, the results of the loading control weren't as explicit as expected.

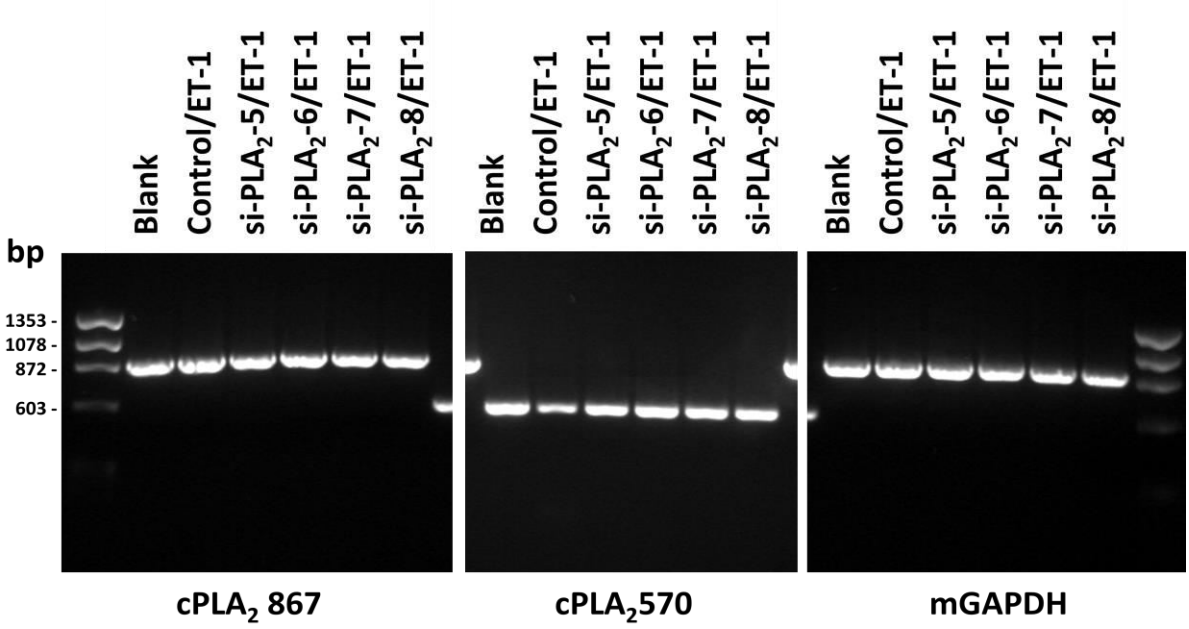

Figure 7: RT-PCR analysis of cPLA$_2$in MC3T3 cells (subculture 21) after transfection with different siRNAs and stimulation with ET-1

After the lysis of MC3T3 cells with RLT buffer, subsequent RNA quantification, reverse transcription and amplification, 5 µl standard and 15 µl of the samples supplemented with 5 µl PCR buffer were loaded onto the gel. The final concentration of the samples was 100 ng/5 µl and 50 ng/5 µl for the mGAPDH loading control. The run was carried out with 1 x TBE buffer at 100 V constantly for 60 minutes.

7.1.2 RT-PCR analysis of cPLA$_2$

RT-PCR analysis shown on figure 7 was prepared using two different primers for cPLA$_2$ with 570 bp and 867 bp, obtained from *Quiagen*. GAPDH was used as loading control. The PCR of cPLA$_2$ for both primers results for each case in one amplification product with the expected size. Unfortunately, there appears to be no difference in the amount of phospholipase mRNA between silenced and non-silenced control, judging from the band intensities from both primer products.

The measurement of Prostaglandin E_2 for all silencing procedures was conducted with gaschromatography and mass spectrometry. To clarify the reproducibility and precise values, each batch was carried out in triplicate. The Y-axis of the diagram is representing the amount of PGE_2 measured in ng/well/2ml.

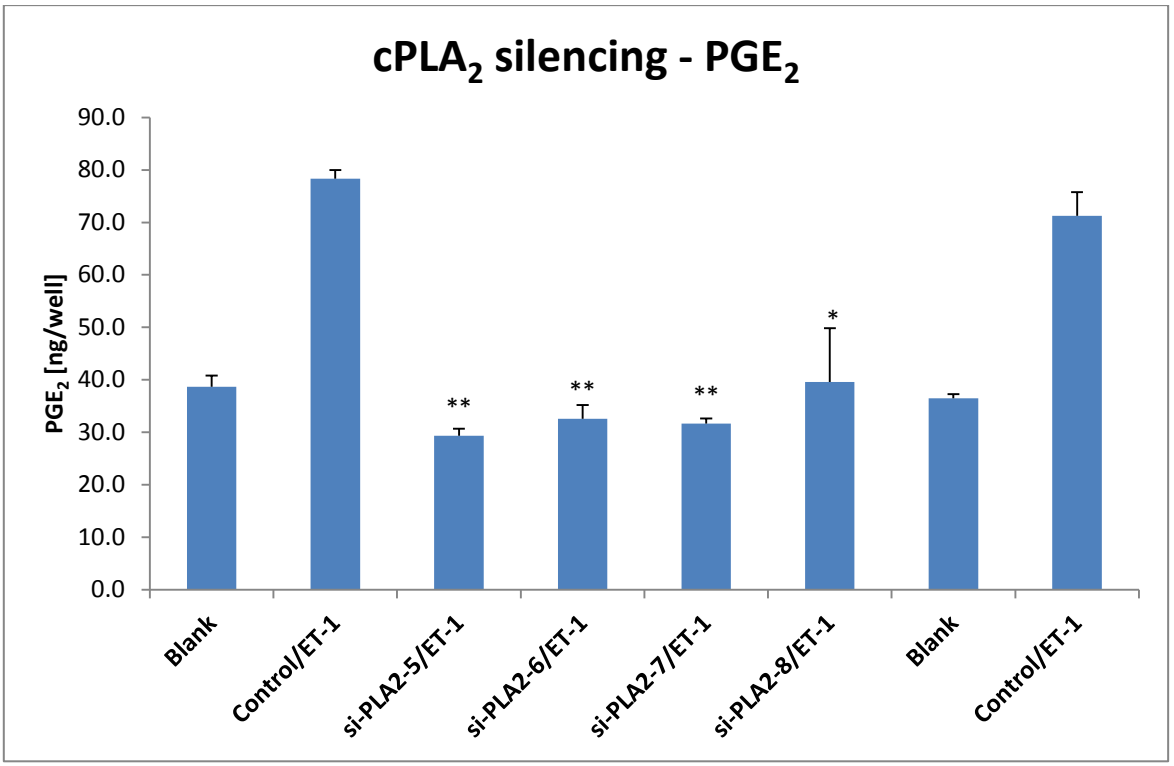

Figure 8: Prostaglandin E_2 release of MC3T3 cells after $cPLA_2$ specific silencing

Cells were seeded (80.000/well/2ml) and left grow to confluence for 24 hours before starting the transfection and stimulation with 50 nM ET-1, respectively. Samples were tested without transfection as control**.** After the primary extraction the organic phase was derivatized with PFBBr and DIPEA. Followed by a MOX extraction step, supernatant was completely dried under a nitrogen stream and dissolved in MSTFA containing 1% TMCS silylation reagent. After a last drying step samples were finally dissolved in n-Hexane containing 1 % BSTFA and measured via GC-MS (negative ion CI). 50µl D_4-PGE_2 (m/z 528) was used as internal standard for the quantification of PGE_2 (m/z 524). **P*< 0.05, ***P*< 0.01, (ANOVA) compared with untreated control.

7.1.3 PGE_2 release after $cPLA_2$ silencing

The amounts of PGE_2 measured by GC-MS and quantified via internal standard concentration showed distinct differences between $cPLA_2$ silenced samples and untreated control. Preparation of the data yielded a diagram which shows that the amount of PGE_2 from the treatment with the si-PLA_2s 5,6 and 7 are on basal activity levels (figure 8). The release of PGE_2 from the si-PLA_2 8 did also work out although not with the same significant values.

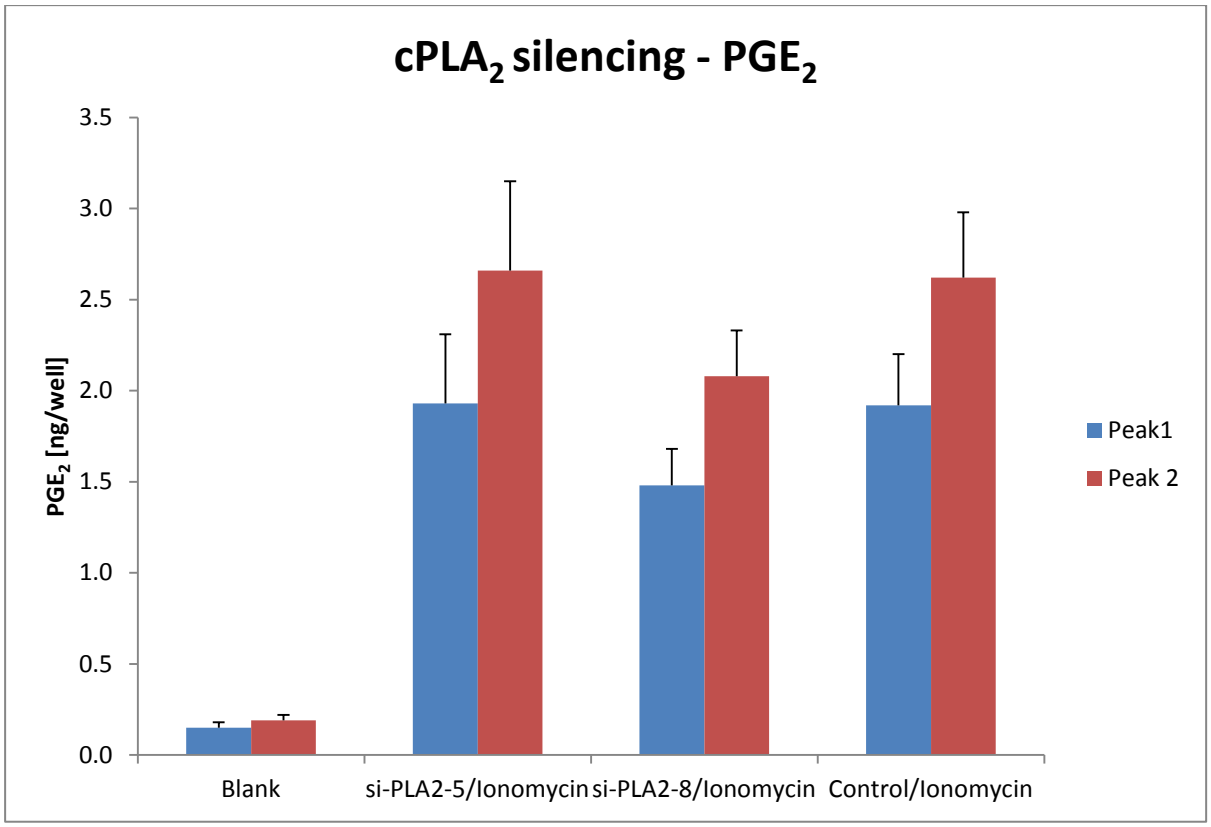

Figure 9: Prostaglandin E_2 levels measured in MC3T3 cells after $cPLA_2$ specific silencing and stimulation with ionomycin

Samples were tested without transfection as control. The effect of ionomycin instead of ET-1 for cell stimulation was tested in this experiment. Therefore cells were incubated for 15 minutes in a medium containing 1.8 mM Ca^{2+} and then stimulated for 30 minutes with the same medium supplemented 2 µM ionomycin. Following steps were equal to the other experiments. After the primary extraction the organic phase was derivatized with PFBBr and DIPEA. Followed by a MOX extraction step, supernatant was completely dried under a nitrogen stream and dissolved in MSTFA containing 1% TMCS silylation reagent. After a last drying step, samples were finally dissolved in n-Hexane containing 1% BSTFA and measured via GC-MS (negative ion CI). 50 µl D_4-PGE_2 was used as internal standard for the quantification of PGE_2.

7.1.4 $cPLA_2$ - Ionomycin

Due to the fact that several stimulation and transfection steps can lead to a significant decrease in cell viability, the stimulation effect of ionomycin was tested. With regard to the total amount, the diagram demonstrates that the amount of PGE_2 from silenced samples and untreated control is nearly equal. The experiments with the Ca^{2+} ionophore ionomycin as supplement decreased the total PGE_2 levels compared with previous measurements. (figure 8, 9)

7.2 PLD1 and PLD2 silencing experiments

After the $cPLA_2$ silencing was successful reproducible, same procedure started with siRNA duplexes specific for PLD1 and PLD2. Because of the lack of Western Blot specific antibodies for each of those PLDs, following experiments focused on the results of RT-PCR and GC-MS PGE_2 measurement.

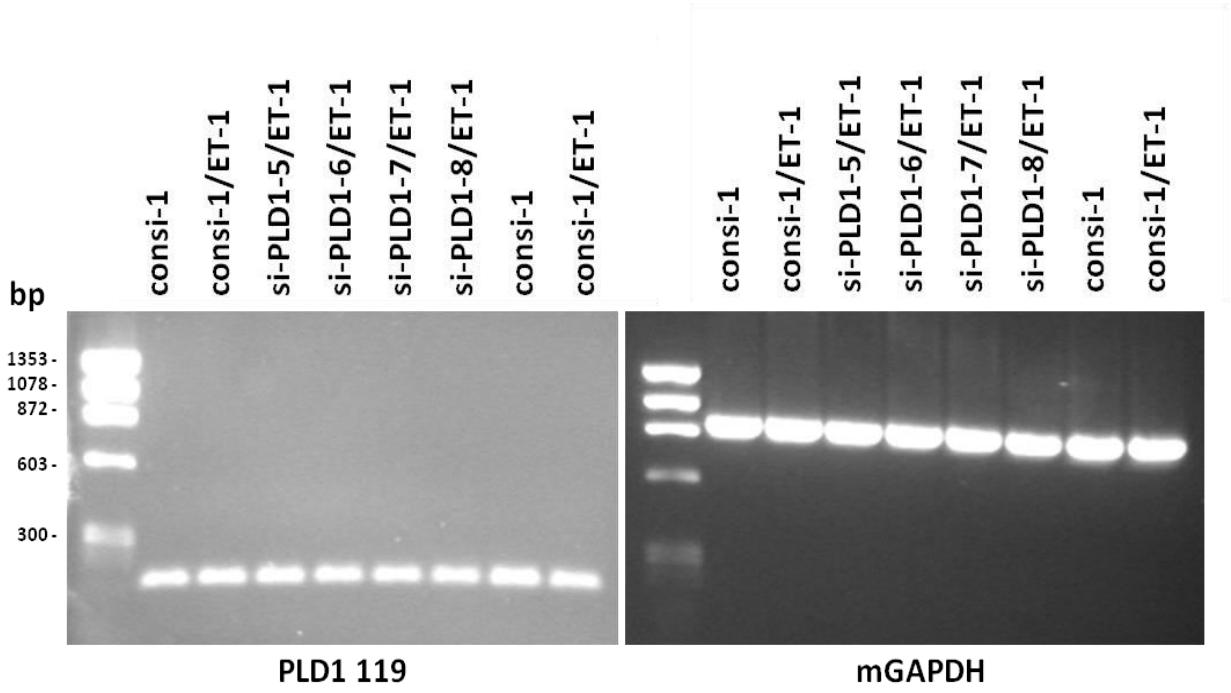

Figure 10: RT-PCR analysis of PLD1 in MC3T3 cells (subculture 33) after transfection with different siRNAs and stimulation with ET-1

After the lysis of MC3T3 cells with RLT buffer, subsequent RNA quantification, reverse transcription and amplification, 5 µl standard, 15 µl of the samples supplemented with 5 µl PCR buffer were loaded onto the gel. The final concentration of the samples was 100 ng/5 µl and 50 ng/5 µl for the mGAPDH loading control. The run was carried out with 1 x TBE buffer at 100 V constantly for 60 minutes.

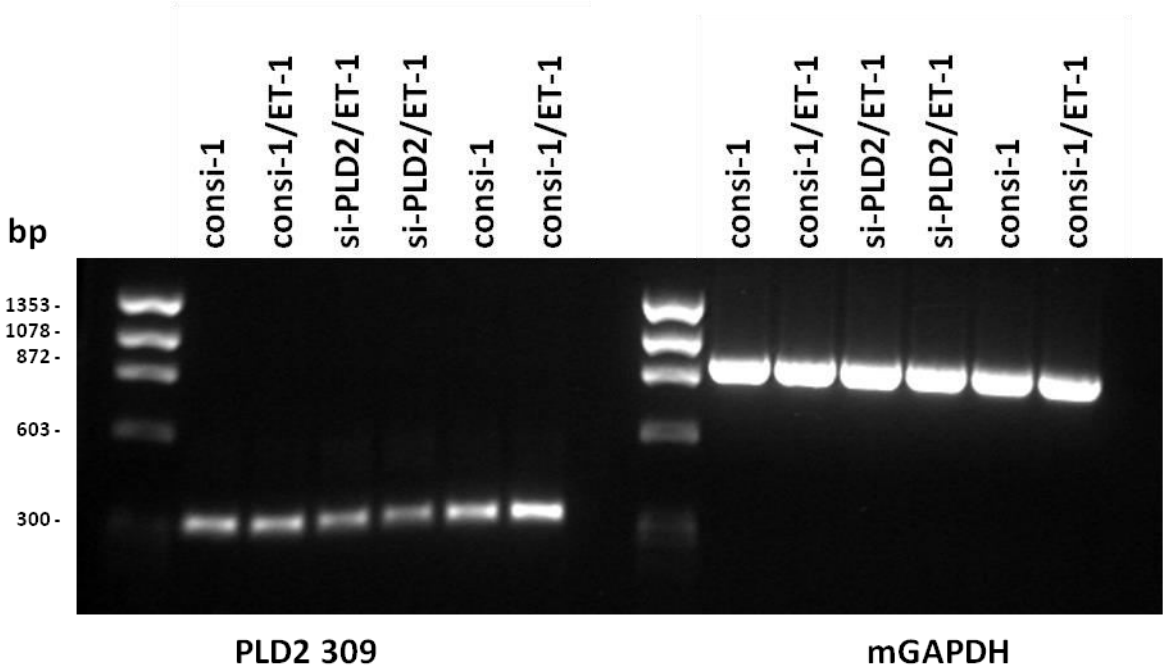

Figure 11: RT-PCR analysis of PLD2 in MC3T3 cells (subculture 29) after transfection with different siRNAs and stimulation with ET-1

After the lysis of MC3T3 cells with RLT buffer, subsequent RNA quantification, reverse transcription and amplification, 5 µl standard, 15 µl of the samples supplemented with 5 µl PCR buffer were loaded onto the gel. The final concentration of the samples was 100 ng/5 µl and 50 ng/5 µl for the mGAPDH loading control. The run was carried out with 1 x TBE buffer at 100 V constantly for 60 minutes.

7.2.1 RT-PCR analysis of PLD1 and PLD2

RT-PCR analysis shown on figure 10 and 11 were prepared using a 109 kDA primer for PLD1 *(Quiagen)* and a 309 kDa primer specific for PLD2 *(Santa Cruz)*. GAPDH was used as loading control for both. The PCR of PLD1 resulted in one amplification product with the expected size as well as the one for PLD2. Where the amplification products of PLD1 showed no differences in their intensities, for PLD2 minimal differences are recognizable, between silenced samples and the untreated control. The loading control showed both times that the RT-PCR did work out.

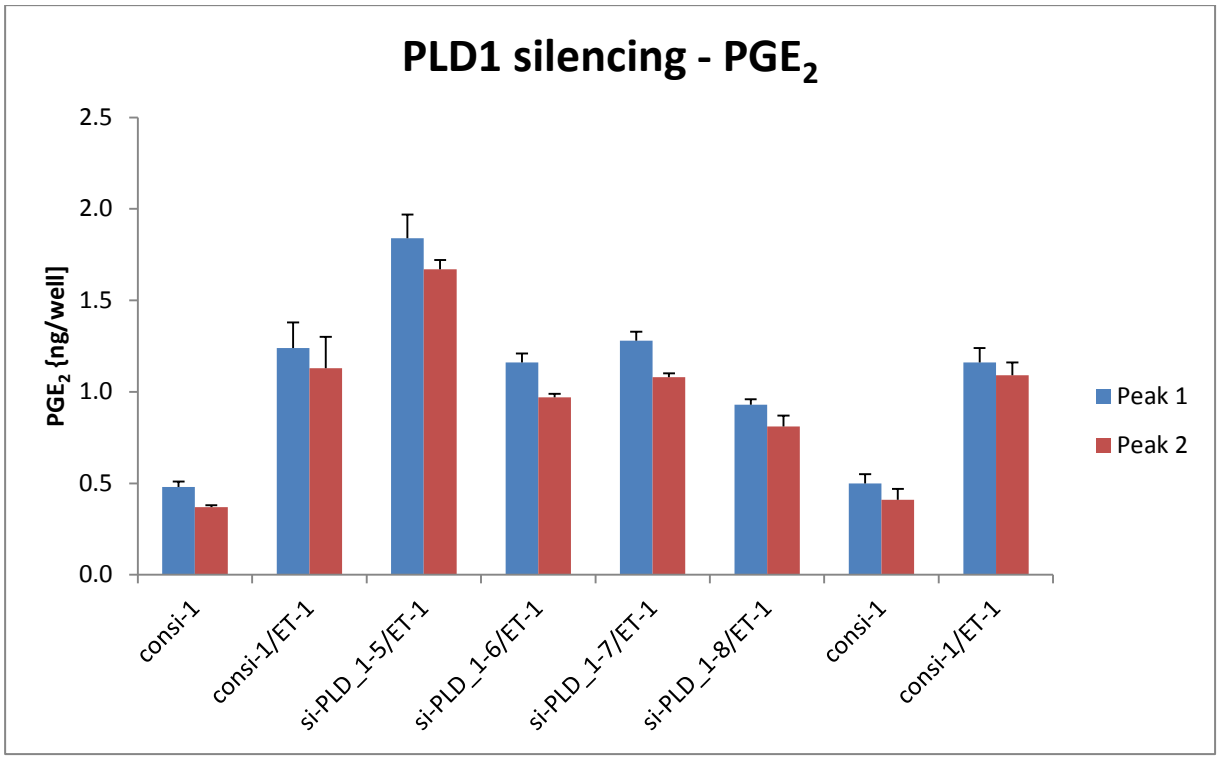

Figure 12: Prostaglandin E_2 release of MC3T3 cells after PLD1 specific silencing

Cells were seeded (80.000/well/2ml) and left grow to confluence for 24 hours before starting the transfection and stimulation with 50 nM ET-1, respectively. Samples were tested without transfection as control. After the primary extraction the organic phase was derivatized with PFBBr and DIPEA. Followed by a MOX extraction step, supernatant was completely dried under a nitrogen stream and dissolved in MSTFA containing 1% TMCS silylation reagent. After a last drying step samples were finally dissolved in n-Hexane containing 1 %BSTFA and measured via GC-MS (negative ion CI). 50 µl D_4-PGE_2 was used as internal standard for the quantification of PGE_2.

7.2.2 PGE_2 release after PLD1 silencing

The amounts of PGE_2 measured by GC-MS and quantified via internal standard concentration showed various differences for all of the tested siRNAs specific for PLD1 (figure 12). The values of si-PLD 6 and 7 silenced samples were as high as those of the untreated controls. Whereas the PGE_2 levels of si-PLD 5 were atypically high. Only the release of PGE_2 from the si-PLA_2 8 silenced samples worked out although on no significant values.

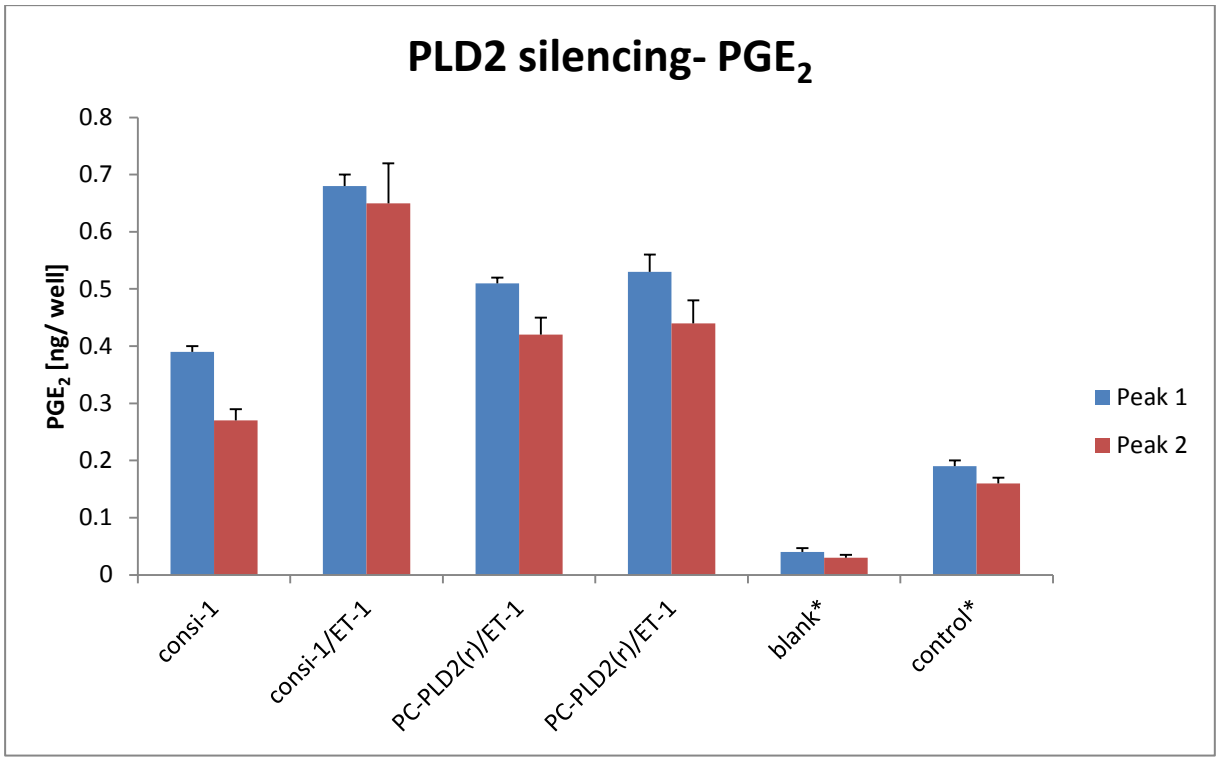

Figure 13: Prostaglandin E_2 release of MC3T3 cells after PLD2 specific silencing

Cells were seeded (80.000/well/2ml) and left grow to confluence for 24 hours before starting the transfection and stimulation with 50 nM ET-1, respectively. Samples were tested without transfection as control. After the primary extraction the organic phase was derivatized with PFBBr and DIPEA. Followed by a MOX extraction step, supernatant was completely dried under a nitrogen stream and dissolved in MSTFA containing 1% TMCS silylation reagent. After a last drying step samples were finally dissolved in n-Hexane containing 1% BSTFA and measured via GC-MS (negative ion CI). 50 µl D_4-PGE_2 was used as internal standard for the quantification of PGE_2.

7.2.3 PGE_2 release after PLD2 silencing

The amounts of PGE_2 measured by GC-MS and quantified via internal standard concentration showed a decrease for the tested siRNA specific for PLD2. The values of PC-PLD2(r) silenced samples, accomplished in duplicate were significant lower than those of the controls without silencing. *Batches of blank and control were accomplished with no transfection, to see if there is a general loss of cell viability because of the treatment (figure 13).

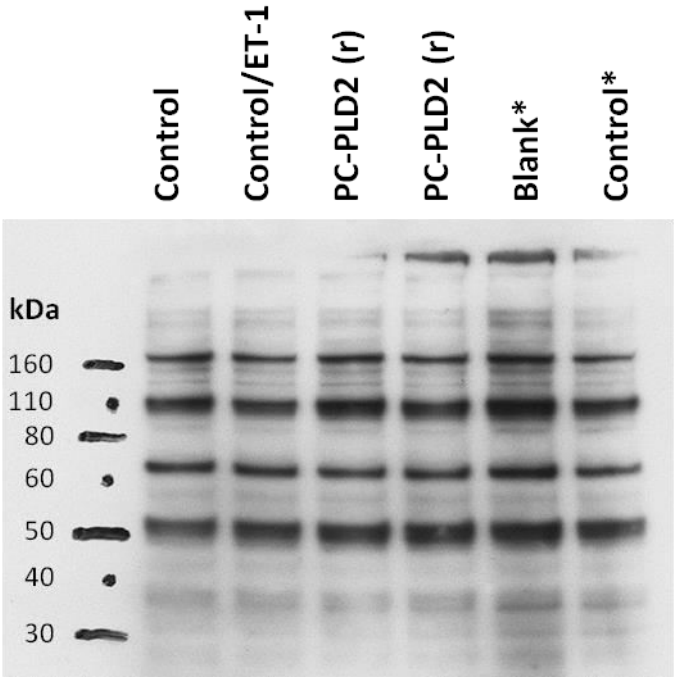

Figure 14: Western Blot analysis of PLD2 in MC3T3 cells after transfection with different siRNAs and stimulation with ET-1

30 µl MC3T3 cell lysate was loaded onto the gel from untreated cells (blank) and after transfection with a final concentration of 100 nm PLA2g4a_5-8 ($cPLA_2$) and stimulation with 50 nm ET-1 for 30 minutes. Control was treated with ET-1 without any transfection, respectively. Subsequent treatment of the membrane with PLD2 primary antibody revealed a band at approximately 117 kDa. β-actin was used as loading control.

7.2.4 Western Blot analysis of PLD2

Cell lysate for Western Blot analysis was collected from untreated cells and after treatment with siRNA duplexes. In figure 14 the Western Blot of MC3T3-E1 cells using PC-PLD2 antibody sc-48370 (V20) *(Santa Cruz)* is shown. Unfortunately, the Western Blot experiments did not yield explicit results. Because of the large amount of bands in the blot, it was not either possible knowing which of those is the one of interest, nor if there is any difference between band intensities. Due to the fact that there is a lack of clean antibodies for PLD2 as well as for PLD1, no further Western Blot experiments were started. *Batches of blank and control were accomplished with no transfection, to see if there is a general loss of cell viability because of the treatment.

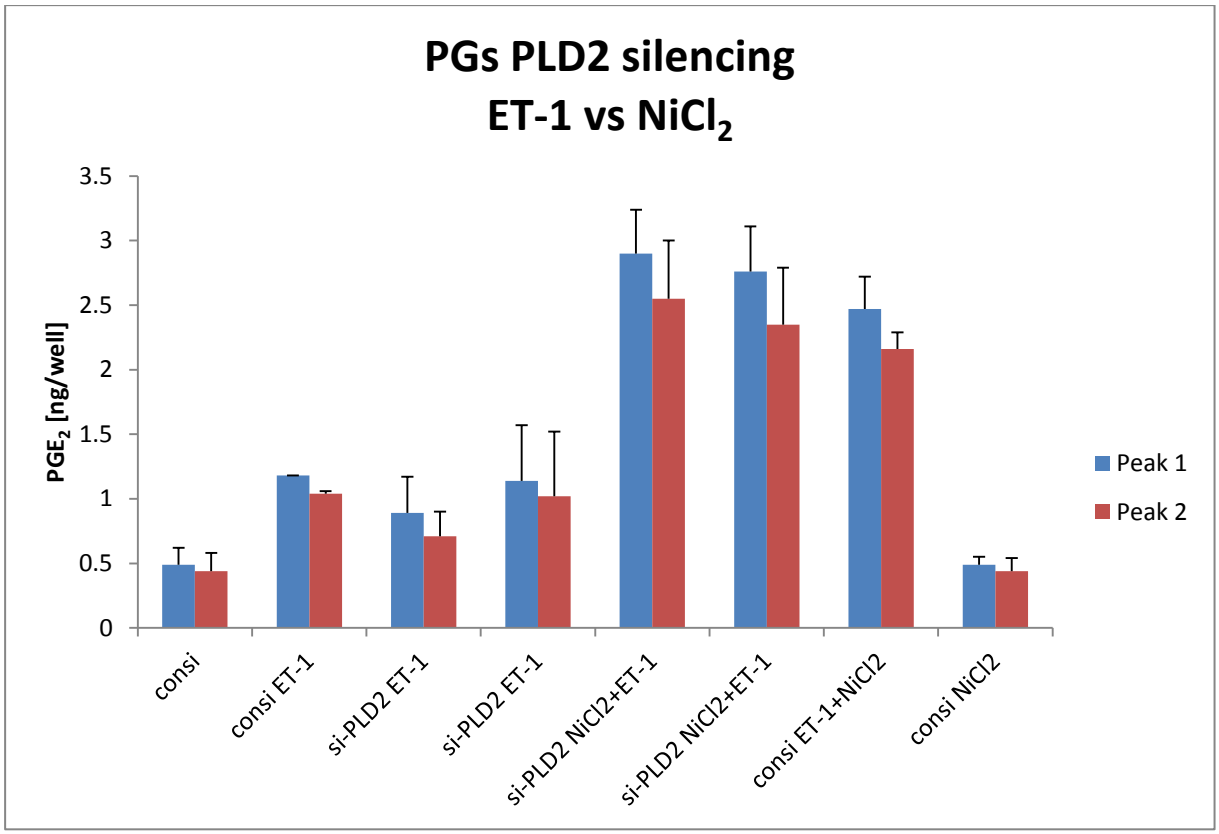

Figure 15: PGE_2 measurements after PLD2 silencing and stimulation with ET-1 and $NiCl_2$

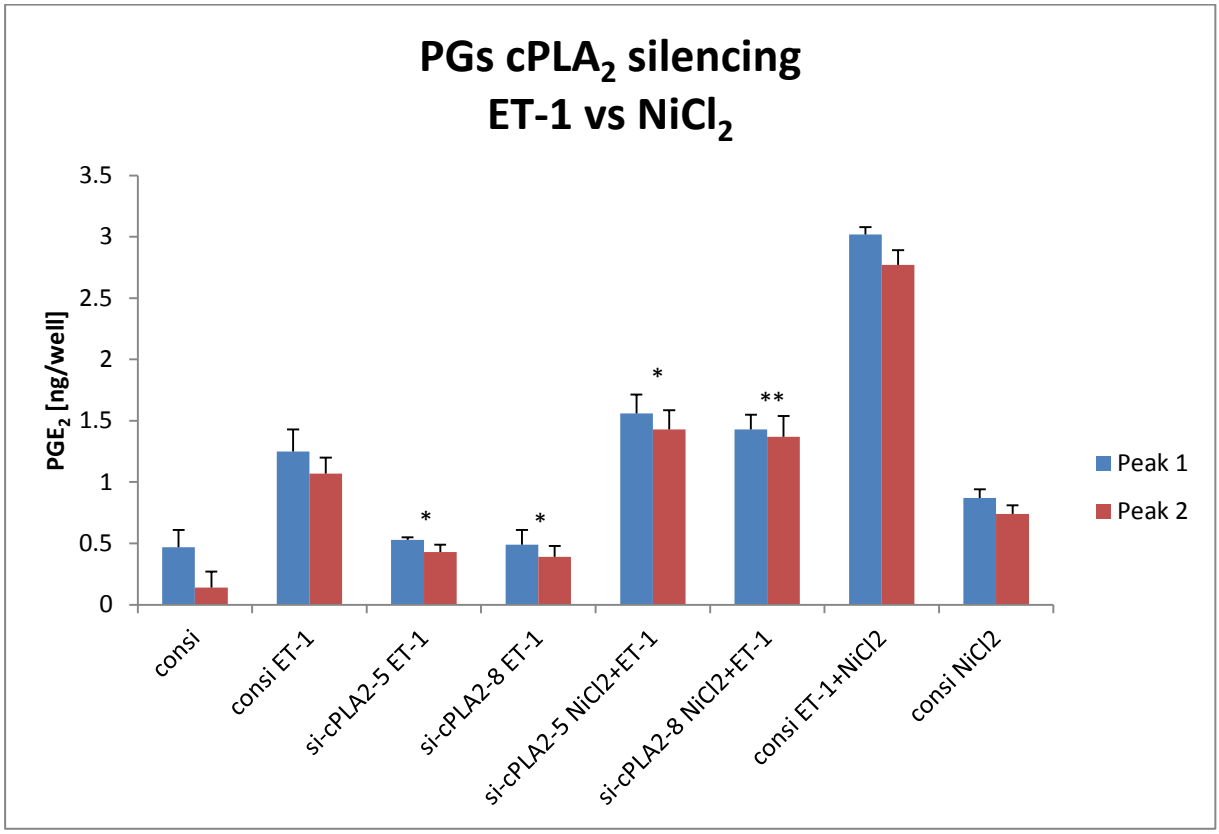

Figure 16: PGE_2 measurements after $cPLA_2$ silencing and stimulation with ET-1 and $NiCl_2$

7.2.5 Stimulation with ET-1 and $NiCl_2$

The Prostaglandin E_2 release of MC3T3 cells after PLD2 and $cPLA_2$ specific silencing and stimulation with either ET-1 or ET-1 and $NiCl_2$ was tested (figure 15 and 16). Cells were seeded (80.000/well/2ml) and left grow to confluence for 24 hours before starting the transfection and stimulation with either 50 nM ET-1 or in combination with 4 mM $NiCl_2$ buffer, respectively. Samples were tested without transfection as control for both conditions. After the primary extraction the organic phase was derivatized with PFBBr and DIPEA. Followed by a MOX extraction step, supernatant was completely dried under a nitrogen stream and dissolved in MSTFA containing 1% TMCS silylation reagent. After a last drying step samples were finally dissolved in n-Hexane containing 1% BSTFA and measured via GC-MS (negative ion CI). 50µl D_4-PGE_2 was used as internal standard for the quantification of PGE_2. $*P< 0.05$, $**P< 0.01$, (ANOVA) compared with untreated control.

The results of PLD2 specific silencing and stimulation with ET-1 in combination with 4 nm $NiCl_2$ containing buffer showed a significant increase of PGE_2 levels compared with the ET-1 buffer alone (figure 15). However, the measurements of silenced samples and untreated controls were nearly equal for both types of stimulation. Suggesting that the silencing of PLD2 did not work out as expected. In contrast to the data of $cPLA_2$ silencing shown in figure 16, all silenced samples showed a significant decrease in PGE_2 levels, and an increase after $NiCl_2$ treatment. This applies equally to both controls.

8 Discussion

The primary intention of this work was to examine the effects of silencing of PLD1, PLD2 and $cPLA_2$ on ET-1 mediated PGE_2 synthesis in MC3T3-E1 cells. PGE_2 is known to regulate multiple aspects of inflammation and multiple functions of different immune cells, thus it is the major prostaglandin produced in osteoblastic cells. Because of the fact that the extraction of low levels of eicosanoids is variable, the use of an internal standard was essential for quantification of the analyte by gas chromatography and mass spectrometry.

The preliminary Western Blot results of the silencing experiments with siRNA duplexes specific for $cPLA_2$ (figure 6) showed that there was a difference between the intensities of silenced and untreated samples. The obtained data indicated a success of silencing on protein level, but could not be confirmed by the results of the RT-PCR analysis using the same samples (figure 7). Due to the fact that if there are visible effects in Western Blot, same results must be verifiable on DNA level, these findings were controversial. The only explanation, yielding those results, could be that the sensitivity and specificity of the chosen method was not high enough, to detect minimal silencing effects on DNA level. To confirm this hypothesis, further experiments need to be performed using e.g. real-time-PCR, which is known to have a greater sensitivity and reproducibility. The evaluation of the measurements of prostaglandin E_2 release of MC3T3 cells after $cPLA_2$ specific silencing correlated exactly with those of the Western Blot (figure 8, 6). Compared with the controls, PGE_2 production was diminished after transfection with the four tested siRNA duplexes. Furthermore standard deviation confirmed that the batches carried out in triplicate showed moderate to no fluctuations. Similarly a significance of $^{**}P < 0.01$ has been reached. However, PGE_2 levels of si-PLA2-8 were not as explicit as the other ones, suggesting that the sequence of the si-RNA had a lower compatibility to the sequence from the gene of interest. In consideration of the experiments, it could be confirmed that cPLA2 is one of the major players in arachidonate liberation and furthermore prostaglandin E_2 formation. As well as ET-1 stimulation leads to an increasing stimulation and activation of $cPLA_2$.

Beside those findings, studies of Zhi-Hua et al suggested that arachidonic acid release is induced with calcium ionophores such as ionomycin. Due to their investigations, that ionomycin induces calcium mobilization and promotes large amounts of arachidonic acid, for the phase of stimulation endothelin-1 was replaced by ionomycin (figure 9). With regard to the total amount of PGE_2, the diagram demonstrates that the amount from silenced samples and untreated control was nearly equal. As well as the total PGE_2 levels were decreased, compared with previous measurements. Therefore, for subsequent experiments ionomycin as stimulation agent was discarded.

After the successful reproducibility of $cPLA_2$ experiments, the same procedure was started with siRNA duplexes specific for PLD1 and PLD2. Figure 10 and 11 demonstrate RT-PCR analysis of PLD1 and PLD2 in MC3T3 cells, after transfection with the specific siRNA and ET-1 stimulation. Compared to the mGAPDH loading control, only PLD2 samples showed a decrease in band intensities after silencing. As already mentioned for cPLA, this may reflect the fact that sensitivity and specificity of RT-PCR was not high enough to detect changes in such small amounts of the corresponding analyte. Because of the lack of Western Blot specific antibodies for each of those PLD1 as well as PLD2 (figure 14), the success of silencing and stimulation could only be verified by means of PGE_2 levels from the GC-MS measurements (figure 12, 13). The amounts of PGE_2 showed various differences for all tested siRNAs specific for PLD1. Where si-PLD_1-6 to 8 showed nearly equal amounts in prostaglandin formation compared to the control, levels of si-PLD_1-5 were atypically high. From these measurements alone, it neither can be said that PLD1 carries a part in the arachidonate liberation nor that it plays no role at all. The variations in results caused by the silencing through different siRNAs, could be either explained by an imperfect sequence specifity or that the silencing did work out, but PLD1 had no influence on arachidonate liberation and prostaglandin formation. These hypotheses can only be clarified in combination with accurate results from Western Blot and polymerase chain reaction, so it is necessary to start further experiment e.g. using qPCR as silencing detection method. A silencing experiment performed with real-time PCR can be one of the most sensitive, efficient, fast, and reproducible methods of measuring gene expression.

In contrast to PLD1, GC-MS results after PLD2 silencing showed a minor decrease of PGE_2 levels (figure 13). An explanation for this might be that the silencing of PLD2 did work out as expected, and phospholipase D2 carries a minimum proportion in arachidonate release and PGE_2 formation. On the other hand it must be mentioned, that the differences between silenced samples and untreated control showed no statistical significance, compared to the total measured amount of PGE_2. Unfortunately it was not possible to obtain other siRNA duplexes, specific for PLD2, to precise the outcome of the experiments. The problem occurred also in the selection of antibodies against PLD2 (figure 14).

Through the results obtained, subsequent experiments focused on the effect of $NiCl_2$ on prostaglandin E2 levels. Therefore PLD2 and cPLA2 specific silenced samples where either stimulated with endothelin-1 or in combination with 4mM $NiCl_2$, added to the buffer. As shown in figure 15 and 16, the effect of ET-1 and $NiCl_2$ together leads to nearly twice as high PGE2 levels, for both, PLD2 and $cPLA_2$ silenced samples. Those finding suggested that $NiCl_2$ supplemented to the buffer, appears to activate a pathway that is independent from endothelin-1 induced calcium mobilization, described as

the nickel effect.[72,73] Irrespective of this successful outcome, $cPLA_2$ showed the same results as described before. After silencing and stimulation of cytosolic phospholipase A_2, PGE_2 levels were significantly decreased, compared with the untreated control. Whereas the PLD2 silenced samples control showed nearly equal amounts of prostaglandin E_2, as those of the control.

The aim of the thesis was to examine if there exists interplay between the pathways that are probably involved in the endothelin-1 induced arachidonate liberation and prostaglandin E_2 formation in MC3T3 cells. Due to the fact that each result of the three chosen analysis methods, including Western Blot, RT-PCR and GC-MS measurement, is necessary to obtain a correct and reproducible statement, further experiments need to be performed. Based on this it can absolutely certain be said, that independently from PLD2 and PLD1, $cPLA_2$ is the big player in AA release and PGE_2 formation. By the means of our experiments, the role of PLD1 and PLD2 can be neither précised or confirmed nor refused. Continued experiments on this matter need to include the improvement of clean antibodies for Western Blot and a qPCR method with higher sensitivity and spectivity.

[72] {Leis 1994 #23}
[73] {Leis 2016 #24}

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10 List of Figures

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