Phosphatidylinositol Transfer Proteins in Cell Survival and Apoptosis

Fosfatidylinositol transport eiwitten in celdood en overleving

(met een samenvatting in het Nederlands)

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“The joy of discovery is certainly the liveliest
that the mind of man can ever feel.”
- Claude Bernard (1813-1878) -

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Contents

Abbreviations

2AG	2-arachidonoylglycerol
AA	arachidonic acid
AEA	anandamide (arachidonoylethanolamide)
BSA	bovine serum albumine
cAMP	cyclic adenosinemonophosphate
CB	cannabinoid
CREB	cyclic AMP response element (CRE)-binding protein
CM	conditioned medium
DAG	diacylglycerol
DBB	DMEM containing 0.1 % bovine serum albumin
DGL	diacylglycerol lipase
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
GPI	glycerophosphatidylinositol
I(1)P	inositol 1-phosphate
LysoPI	lysophosphatidylinositol
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor-κB
NsL-TP	non-specific lipid transfer protein
OEA	oleoylethanolamide
PC	phosphatidylcholine
PC-TP	phosphatidylcholine transfer protein
PEA	palmitoylethanolamide
PG	prostaglandin
PI	phosphatidylinositol
PI(3)P	phosphatidylinositol 3-phosphate
PI(3,4,5)P_3	phosphatidylinositol 3,4,5-triphosphate
PI 3K	phosphatidylinositol 3-kinase
PI(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
PI-TP	phosphatidylinositol transfer protein
PKA	protein kinase A
PKB	protein kinase B (identical to Rac and Akt)
PKC	protein kinase C
PLA	phospholipase A
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PS	phosphatidylserine
SM	sphingomyelin
THC	tetrahydrocannabinol
TLC	thin layer chromatography
TNFα	tumor necrosis factor α

Chapter 1

Introduction

Introduction

General

Phospholipid transfer proteins occur in a wide range of organisms, such as mammals, plants, yeast and fungi and are characterized by the ability to catalyze the transfer of phospholipids between membranes *in vitro* [1]. Plasma phospholipid transfer proteins belong to the lipid binding protein family including the cholesteryl ester transfer protein, the bactericidal permeability increasing protein and the lipopolysaccharide-binding protein [2, 3]. Cytosol phospholipid transfer proteins were first detected in 1968 when Wirtz and Zilversmit observed that a small protein in the membrane-free rat liver cytosol was responsible for the transfer of radiolabelled phospholipids between microsomes and mitochondria *in vitro*. Since then several classes of small cytosolic proteins have been identified from mammalian tissues, which are able to transfer a wide range of phospholipids including sterols. The three major classes are: (a) the non-specific lipid transfer protein (nsL-TP), also designated as sterol carrier protein 2 (SCP-2), (b) phosphatidylcholine transfer proteins (PC-TP's) and (c) phosphatidylinositol transfer proteins (PI-TP's). Despite the lack of a primary structure homology, the 3D structure of all these proteins shows a distinct hydrophobic cavity suited for lipid binding [4-7]. The distinction between these phospholipid transfer proteins lies within their affinity for specific phospholipid headgroups. NsL-TP, a 13 kDa peroxisomal protein [8, 9] was shown to be able to transfer a wide variety of lipids such as phospholipids, glycosphingolipids as well as cholesterol and fatty acyl-CoAs *in vitro* [10]. With the ability to bind branched chain fatty acyl-CoAs it is proposed to be involved in the β-oxidation of these fatty acids in peroxisomes, where nsL-TP also constitutes the C-terminal part of the 58-kDa peroxisomal 3-oxoacyl-CoA thiolase [8, 9, 11]

Phosphatidylcholine transfer protein (PC-TP) was first isolated in 1972 from bovine liver and solely transfers phosphatidylcholine between membranes *in vitro* [1, 12]. Although knockout mice lacking PC-TP did not show a noticeable change in phenotype, the protein was found to be involved in the size regulation and the hepatic uptake of high-density lipoproteins [13] [14].

Phosphatidylinositol transfer protein (PT-TP) was initially isolated in 1974 [15, 16]. *In vitro* this protein has transfer activity for phosphatidylinositol (PI) and to a lesser extent for phosphatidylcholine (PC) and sphingomyelin (SM) [1, 17-19]

History

Since the discovery and purification, a number of isoforms and proteins containing a PI-TP-like domain have been detected, among which the low molecular weight PI-TPα and PI-TPβ and the high molecular weight, membrane associated Nir/RdgB family. The PI-TP homologue in yeast is Sec14p. PI-TP isoforms are highly conserved in mammalian tissue but also occur in plants, yeast, insects and fungi strains [1, 20].

Table 1. Diversity of PI-TP (adopted from Lev 2004 and Cockcroft 1997)

Class	Name	Species	Size (amino acids)
Mammalian PI-TP	PITPα	human, rat, mouse, rabbit	270, 271
	PITPβ	rat	271
	Nir1	Human, rat	974, 931
	Nir2	Human, mouse	1244, 1243
	Nir3	Human, mouse	1349, 1281
	MrdgBh	human	332
Yeast PI-TP	$SEC14p^{SC}$	Saccharomyces cerevisiae	303
	$SEC14p^{KL}$	Kluyveromyces lactis	301
	$SEC14p^{YL}$	Yarrowia lipolytica	497
	$SEC14p^{CA}$	Candida albicans	301
Fungi PI-TP	Mucor mucedo	Zygomycetes	24 kDa
	Neurospora crassa	Ascomycetes	19 kDa
	Asperpergillus oryzae	Deuteromycetes	19 kDa
Other PI-TP	rdgB	Drosophila	1054
	pl-RdgB	zebrafish	942
	Rdgb_Ag	A. gambiae	1203
	M01F1.7	C. elegans	1034

In mammals two soluble, low molecular weight isoforms have been identified (i.e. PI-TPα and PI-TPβ), the 271 amino acids residues of which are 77% identical and 94% similar [21, 22]. In addition, recently a novel PITPβ isoform with a naturally occurring Ser262Gln polymorphism was identified [23, 24]. Studies on mRNA levels showed that PI-TPα and PI-TPβ mRNA are present in all tissues investigated including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas [25]. Highest PI-TPα mRNA levels were detected in pancreas and brain, whereas the highest PI-TPβ mRNA level was detected in the liver [25]. In a similar study, high levels of PI-TPα and PI-TPβ mRNA were detected in rat brain, and very high PI-TPα mRNA levels in rat testes [21, 26]. PI-TPα and PI-TPβ protein levels were determined in various mice organs by means of Western blot analysis using highly specific antibodies against PI-TPα and PI-TPβ.

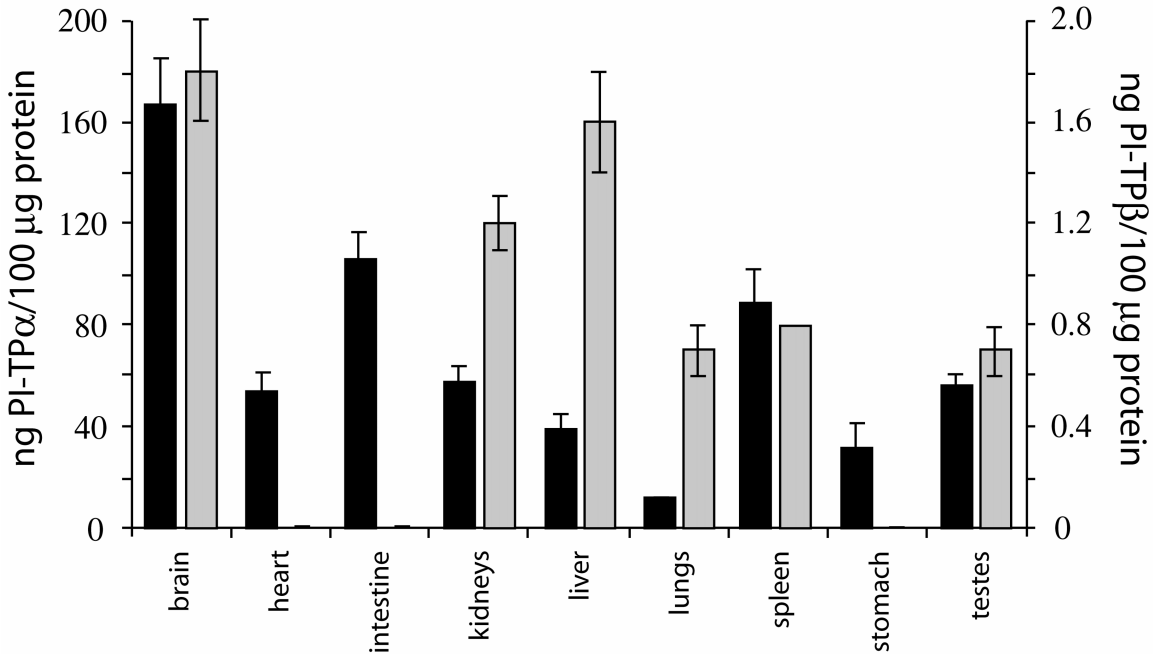

Figure 1. The protein level of PI-TPα (black bars) and PI-TPβ (gray bars) in mouse tissue.

In agreement with mRNA, PI-TPα was detected in all tissues investigated, with highest levels in brain, intestine and spleen. The highest PI-TPβ levels were measured in brain, liver and kidney. No PI-TPβ was detected in heart, intestine and stomach. This may be

due to the very low levels of PI-TPβ which are approximately 50-100 fold lower than PI-TPα levels [27].

Isolation, characterization and cellular localization

When PI-TPα was first isolated from bovine brain two charge isomers where distinguished. Both forms had an approximate molecular weight of 35 kDa, yet they differed in their isoelectric point. This variation was caused by the phospholipid accommodated in the hydrophobic cavity of the proteins. The isoform with an isoelectric point of 5.5 contained the negatively charged PI, whereas the isoform with an isoelectric point of 5.7 was found to carry the zwitterionic PC [28]. In addition to binding PI and PC, PI-TPα was shown to mediate the exchange of both lipids between membranes *in vitro* although at a different rate (PI>PC)[29, 30]

In an attempt to identify a phosphorylated form of PI-TPα in bovine brain and in NIH3T3 cells, a new more acid isoform was discovered, denoted PI-TPβ [19, 22]. This isoform, which cross-reacted with antibodies raised against peptides representing PI-TPα epitopes, had a molecular weight of 36 kDa and an isoelectric point of 5.4 [19, 22]. In addition to PI and PC, this isoform also transfered sphingomyelin. This finding was in line with a PI-TP expressing sphingomyelin transfer activity in chicken liver [31]. In a recent study it was shown that the SM transfer activity depends on the C-terminal segment of PI-TPβ, which is substantially different from that of PI-TPα [24]. Analysis of the cDNAs encoding mouse, rat and human PI-TPα and PI-TPβ showed that both isoforms were completely conserved between mammalian species with a amino acid sequence identity of about 99% [30, 32]. Localization studies by immunofluorescence and by microinjection of fluorescently labeled purified PI-TPα and PI-TPβ into intact fetal bovine heart endothelial cells have shown that PI-TPα is mainly localized in the nucleus and in cluster-like structures in the cytosol whereas PI-TPβ was shown to be predominantly associated with de Golgi system [19, 33].

Two-dimensional tryptic peptide mapping revealed that PI-TPα had one minor protein kinase C dependent phosphorylation site (i.e. Ser-166) whereas PI-TPβ had one

minor phosphorylation site (i.e. Ser-165) and one major phosphorylation site (i.e. Ser-262). *In situ*, PI-TPβ seems to be constitutively phosphorylated at Ser-262 (more than 80%) [18]. Upon incubation with the PKC inhibitor GF 109203X, a shift from the phosphorylated to the non-phosphorylated form was observed. Using a clone in which Ser-262 was replaced with an alanine, the mutant PI-TPβ was released from the Golgi, strongly suggesting that phosphorylation of the Ser-262 was a prerequisite for PI-TPβ to be associated with the Golgi system [18]. In a current study a novel PITPβ isoform with a naturally occurring Ser262Gln polymorphism was also found to be localized to the trans-Golgi network. In addition incubation with GF 109203X had no effect on the Golgi localization of PI-TPβ thereby contradicting previous findings [23, 24].

Cellular functions of PI-TPα and PI-TP β

Extensive studies on the cellular function of PI-TP's have been performed using reconstituted (Golgi) membrane systems, permeabilized cells and cells in which the expression has been manipulated. In addition, PI-TPα knockout mice and mice with a reduced level of PI-TPα (vibrator mouse) have been studied.

Vesicle formation, fusion and secretion

Vesicular traffic in eukaryotic cells consists of two types of membrane reorganization. First, vesicles are formed by budding from donor membranes (e.g. trans-Golgi network). Secondly, the released vesicles must fuse with acceptor membranes (e.g. plasma membrane). Several cytosolic proteins involved in budding and fission have been identified. Using a cell-free system derived from a neuroendocrine cell line containing a highly purified trans-Golgi network, it was shown that PI-TPα and PI-TPβ were able to facilitate the formation of constitutive secretory vesicles and immature secretory granules from the trans-Golgi network. In addition to being active in vesicle formation, PI-TP was also one of the three factors present in rat brain cytosol responsible for the fusion of secretory granules with plasma membrane. Moreover it was shown, by using

permeabilized (semi-intact) cytosol-depleted PC12 cells, that PI-TPα was required for the ATP-dependent priming of Ca^{2+}-regulated secretion of noradrenaline (norepinephrine). [34, 35]. In a similar study using cytosol-depleted HL60 cells it was shown that recombinant PI-TPα and PI-TPβ both restored GTPγS-dependent protein secretion [36].
In line with PI-TP being able to stimulate PIP_2 synthesis in cytosol-depleted HL60 cells, a type I phosphatidylinositol-4-phosphate 5-kinase (PIP-5-kinase) was found to be involved in the budding process [36, 37]. In the proposed model, PI-TPα carries PI to sites of phosphorylation, thereby promoting the synthesis of PIP_2 [34].
In these systems Sec14 protein was able to substitute for the mammalian PI-TP [37]. Since Sec14p has no sequence homology with mammalian PI-TP it appears that the transfer of PI is sufficient for the proposed cellular functions.

Phospholipase C mediated inositol lipid signaling

A role for PI-TP in PIP_2 formation was inferred from the observation that PI-TP was part of a multi-enzyme complex, which furthermore consisted of the epidermal growth factor-receptor (EGF), phospholipase C-γ and PI-4 kinase. Increased levels of PI(4)P and PIP_2 in cytosol-depleted A431 cells after addition of PI-TP and EGF further supported this claim [38]. This study and early work by Thomas *et al.* formed the starting point of extensive studies on the role of PI-TP in phospholipase C mediated inositol signaling [19, 39-42]. In this signaling process phospholipase C is a key enzyme by catalyzing the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to $I(1,4,5)P_3$ and diacylglycerol (DAG). IP_3 mediates the rapid mobilization of Ca^{2+} from the endoplasmic reticulum (ER) by binding to specific intracellular receptors, whereas diacylglycerol stimulates protein kinase C, resulting in the phosphorylation of intracellular proteins [43].
The proposed function of PI-TP in inositol lipid signaling as a substrate provider for PLC was supported by the finding that PI-TP dictates the rate of IP_n production by being essential for PIP_2 production [41]. A shortcoming of these studies is that the PI-TP-dependent activation of PLC is estimated by measuring the incorporation of [^{3}H]-inositol in the total water-soluble IP_n fraction. However, this fraction contains at least 20 inositol metabolites of which only $I(1,4,5)P_3$, $I(1,4)P_2$ and I(4)P are produced after activation of

PLC. The model proposed by Cunningham *et al*. in which PI-TP was required as a co-factor for the phosphoinositide-metabolizing enzymes was further questioned when inositol lipid signaling was investigated in turkey erythrocyte ghosts. Currie *et al.* showed that PI-TP did not enhance the initial rate of PI 4-kinase activity but increased the steady-state levels of both PIP and PIP_2 [42]. Hence, it appears that the function of PI-TP in inositol lipid signaling is restricted to PI transport to the site of PIP synthesis and that PI-TP does not catalyze the actual formation of PIP_2 [42].

To date, many different lipids have been shown to be present inside the nucleus, such as chromatin-associated phospholipid, phosphoinositides like PI(4,5)P_2 and PI(5)P acting as nuclear signaling molecules, neutral lipids and cholesterol [44-51]. The discovery of PKC and DAG within the nucleus supported the existence of an intranuclear signaling pathway [52]. Since PI-TPα is localized in the nucleus (see above), it was thought that this transfer protein assists in the nuclear import of PI. However, studies using PI-TP$\alpha^{-/-}$ mouse embryonic fibroblasts demonstrated no difference in the nuclear import of PI [53].

Although emerging evidence shows a profound role for endonuclear phospholipids in cell survival, proliferation, DNA repair and premessenger RNA splicing, numerous questions about phospholipid metabolism in the nucleus and the likely role for PI-TPα remain unanswered to date [46, 48, 54, 55]

PC metabolism

The essential function of yeast PI-TP (Sec14p) is to maintain the vesicle flow from the late-Golgi compartment to the plasma membrane. A temperature-sensitive Sec14-1^{ts} strain, which demonstrates a lethal defect when grown at 37^{o}C, was used to show that the ability of Sec14p to transfer PI and/or PC is directly related to Golgi secretory function and cell viability [56-58]. The mechanism by which the transfer activity of Sec14p affects the viability of yeast cells was elucidated when mutations in enzymes of the cytidine diphosphate-choline (CDP-choline) pathway abolished the dysfunction of Sec14p in the Sec14-1^{ts} strain [59]. Both mutations in genes encoding yeast choline kinase and yeast choline phosphotransferase eliminated the growth and secretory defects caused by PI-TP dysfunction, whereas a mutation in genes encoding enzymes associated with the

methylation pathway for PC biosynthesis did not, revealing a direct connection between PI-TP function and the CDP-choline pathway [59]. It was proposed that the PI/PC composition of a membrane is directly dependent on Sec14p activity. The finding that PC-containing Sec14p inhibited cholinephosphate cytidylyltransferase, a key regulatory enzyme in PC biosynthesis, gave rise to the model in which Sec14p was proposed to act as a sensor for proper PI/PC balance of the Golgi membrane [60, 61]. The finding that mammalian PI-TPβ was able to rescue the temperature-sensitive SEC14-1^{ts} strain suggested a similar PC-regulatory function for PI-TP in mammalian tissues [62]. Studies using WRK-1 rat mammary tumor cells transfected with a PI-TPα cDNA inserted in the antisense direction provided further insight into the involvement of PI-TP in PC metabolism. The antisense WRK-1 clones with a decreased level of PI-TPα (approximately 25%) displayed a decreased incorporation of [^{14}C]choline into PC and all PC metabolites except choline phosphate [63]. It was speculated that the decreased level of PI-TPα affected cholinephosphate cytidylyltransferase, hence affecting PC biosynthesis. On the other hand, reduced levels of PI-TPα had no effect on PI, PI(4)P and PIP_2 biosynthesis [63].

Sphingomyelin metabolism

The role of PI-TPβ in sphingomyelin (SM) metabolism was investigated in mouse fibroblast cells with an increased expression of PI-TPβ (SPIβ cells). Although it was shown that increased PI-TPβ levels had no effect on *de novo* synthesis of SM, it was demonstrated that PI-TPβ plays a profound role in maintaining the steady-state levels of SM in the plasma membrane under conditions where ceramide is formed by incubating SPIβ cells with exogenesis sphingomyelinase. This maintenance-function was abolished by the Ser262Ala mutation. The mechanism by which PI-TPβ regulates the steady-state levels of SM in the plasma membrane while localized at the trans-Golgi network is still unclear [64]. It has been speculated that PI-TPβ may function as a SM sensor at the Golgi, similar to its yeast homologue Sec14p acting as a PC sensor.

Activation of a PLA with affinity for PI

The role of PI-TPα in inositol phospholipids metabolism was studied in NIH3T3 mouse fibroblasts transfected with mouse PI-TPα cDNA, resulting in cells characteristically displaying a 2-3 fold increase in PI-TPα (SPIα cells) [65]. Analysis of the inositol phospholipids and metabolites from SPIα cells labeled to equilibrium with myo-[^{3}H]inositol revealed that the level of glycerophosphoinositol was increased 2-fold, of inositol 1-phosphate (I(1)P) and of inositol 2-phosphate (I(2)P) 5-fold, and of lysophosphatidylinositol (lysoPI) 3-fold when compared with control cells. On the other hand, levels of PI and PI(4,5)P_2 were unaffected. The increased levels of lysoPI strongly suggest a link between PI-TPα and a PLA with affinity for PI. In addition, increased levels of I(1)P, I(2)P and glycerophosphoinositol are characteristic for the degradation of lysoPI by lysoPLA and PLC. In support of these observations, addition of purified PI-TPα to a homogenate of [^{3}H]PI-labeled NIH3T3 cells showed an increased production of lysoPI accompanied by a decrease in PI. Based on these observations it was proposed that PI-TPα delivers PI to PLA.

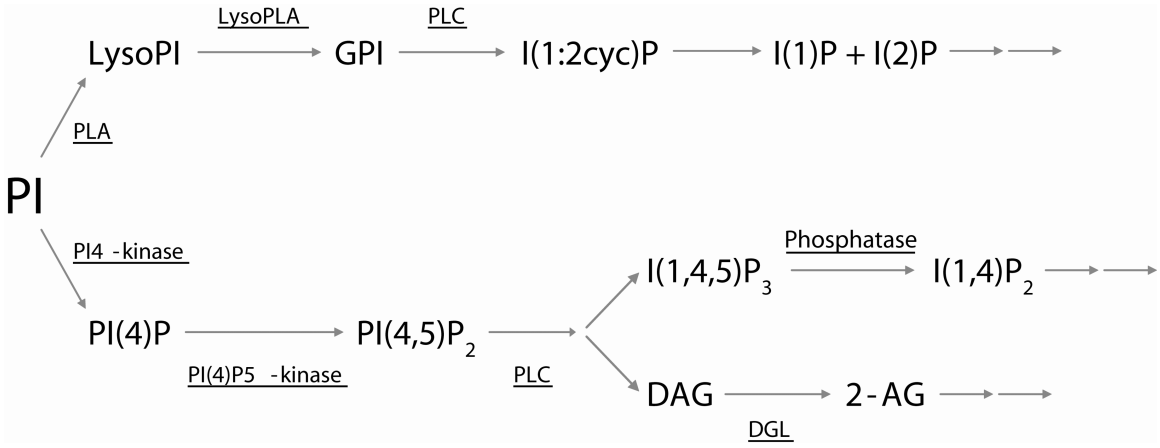

Figure 2. Degradation of phosphoinositol by phospholipase A or phospholipase C. The overexpression of PI-TPα does not change the levels of the PLC-mediated PI metabolites (bottom), whereas the levels of the PLA-mediated metabolites are elevated (top).

The failure to observe any differences in the levels of PI(4)P and PIP_2 in the SPIα cells are difficult to reconcile with the proposed role of PI-TPα in PLC mediated PI-signaling. It should be noted that cells with increased expression of PI-TPβ (SPIβ cells) had no effect on PI-metabolism, which indicates that PI-TPα and PI-TPβ have different functions in the cell.

Regulation of PI-TPα and PI-TPβ

PKC-dependent phosphorylation

The molecular mechanism by which PI-TPs perform their *in vivo* functions and how they are regulated is still poorly understood. In addition, it is still unclear whether the *in vitro* lipid transfer activity is implicated in the *in vivo* function. Sequence analysis of the PI-TPα cDNA revealed the presence of five putative protein kinase C dependent phosphorylations sites: Thr-59, Thr-169, Thr-198, Thr-251, and Ser-166 [30, 66]. Site directed mutations revealed that Ser-166 is the major phosphorylation site in PI-TPα. Stimulation of NIH3T3 cells with PMA induced the translocation of PI-TPα from the cytosol to the Golgi. This translocation was not observed with mutant PI-TPαSer166Ala indicating that phosphorylation of Ser-166 may regulate the relocalization of PI-TPα to the Golgi network. Furthermore it was shown that mutations in Ser-166 completely abolished the PI and PC transfer activity of PI-TPα [67, 68]. Additional information on the regulation of PI-TPα was gathered from structural analysis of the lipid bound form and the apo-form (without a phospholipid bound) [6, 68, 69]. Comparison of the two forms showed that the lipid-carrying form had a closed conformation, whereas the apo-form had a open, more relaxed conformation [69]. Strikingly, in both forms Ser-166 was not exposed to the solvent and hence inaccessible to PKC [70]. It was speculated that upon membrane binding a small loop of PI-TPα inserts into the membrane, giving rise to a slight conformational change that would allow phosphorylation of Ser-166. In this context, it is interesting that the PC-containing form was a better substrate for PKC than the PI-containing form [67]. Apparently, PI bound inside the hydrophobic cavity of PI-

TPα has a negative effect on the availability of Ser-166 for phosphorylation [70]. Both the mutational and structural data demonstrate the essential role of Ser-166 in the function of PI-TPα.

Comparable to the role of Ser-166 in PI-TPα, the transfer activity of PI-TPβ is regulated by Ser-165 [67], which is a minor phosphorylation site in PI-TPβ. In addition, PI-TPβ contains a major phosphorylation site at Ser-262 which is not present in PI-TPα. It appears that *in situ* PI-TPβ is constitutively phosphorylated at this position [18, 24]. It was shown that the replacement of Ser-262 with Ala did not affect the lipid transfer activity of PI-TPβ but did affect the cellular localization. Immunolocalization demonstrated that mutant PI-TPβS262A no longer localized to the Golgi network but distributed throughout the cell. In agreement with this, inhibition of the phosphorylation of PI-TPβ with a PKC inhibitor GF 109203X prevented the targeting of PI-TPβ to the Golgi network [64]. Recent studies question whether the phosphorylation of Ser-262 is essential for PI-TPβ being associated with the Golgi [23, 24]. The significance of Ser-262 in regulating the function of PI-TPβ was confirmed by the finding that NIH3T3 cells expressing mutant PI-TPβS262A display a cell cycle duration and an ability to replenish degraded SM in the plasma membrane comparable to wild type cells.

Receptor interaction

Recently, Xie *et al.* reported the involvement of PI-TPα in neurite guidance and outgrowth in response to netrin-1 [71]. Throughout the wiring of the nervous system neuronal axons extend over a substantial distance to perform their function. Naturally, during the axon outgrowth the growth cone needs to be directed into the proper direction via guidance cues. Netrin is one of these cues and acts on a receptor known as DCC (deleted in colorectal cancer) [72]. Xie *et al.* recently elucidated the mode of activation of DCC by showing that PI-TPα becomes bound to DCC upon netrin-1 binding. This resulted in an increased binding of phosphatidylinositol 5-phosphate (PI(5)P) to PI-TPα, subsequently leading to an increase in the lipid transfer activity of PI-TPα. In their model, PI-TPα regulates the local production of phosphatidylinositides to be substrates in

the PLC pathway, which is important for neurite outgrowth [71, 73]. However these observations are difficult to reconcile with studies by Alb *et al*. showing a normal prenatal development, including brain and motor neuron growth in PI-TPα$^{-/-}$ mice [74].

Proliferation, cell death and cell survival

SPIα cells have an increased growth rate and a higher cell density at saturation when compared to NIH3T3 and mock-transfected cells. The doubling time typically decreased from 21 h for the NIH3T3 cells to 13-14 h for the SPIα clones. Flow cytometric analyses showed that a shorter G_1 phase was the main reason for the rapid proliferation. Cell count at full confluence changed from 0.20 x 10^5 cells/cm^2 for wtNIH3T3 cells to 0.53 x 10^5 cells/cm^2 for the SPIα clones. Further insight into the *in vivo* function of PI-TPα was obtained by reducing the cellular level of PI-TPα. WRK-1 rat mammary tumorcells, which, as a result of transfection, expressed approximately 25% less PI-TPα than control clones showed a decreased growth rate [63]. The precise mechanism by which PI-TPα stimulated cell proliferation remains to be established although the increased growth rate was mainly attributed to the production of specific PI metabolites (see above) [65]. NIH3T3 mouse fibroblasts transfected with cDNA encoding mouse PI-TPβ (SPIβ cells), yielding stable cell lines in which the level of PI-TPβ was increased approximately 16-fold [64, 75]. In contrast to SPIα cells, SPIβ cells have a decreased growth rate with a doubling time of 35 h and are significantly more sensitive towards UV-induced apoptosis compared to wild-type cells [27].

Mice with a reduced level of PI-TPα showed greatly reduced viability. Mice embryos with a complete lack of PI-TPα or severely reduced levels of PI-TPα developed normally during pregnancy but upon birth the pups suffered an early juvenile death. Vibrator mice, which have a 80% reduced level of PI-TPα, display an early onset of progressive action tremor and degeneration of brain stem and spinal cord neurons [76]. Similarly, PI-TPα knockout mice failed to thrive and died within 14 days after birth. Ablation of PI-TPα function resulted in spinocerebellar disease characteristics, hypoglycemia, and intestinal steatosis [74]. From this it was proposed that PI-TPα played a role in signalling pathways

connected to trafficking of luminal lipid cargo from the endoplasmatic reticulum and in glucose homeostasis. PI-TPβ gene ablation failed to give viable embryonic stem cells [75].

Aims and outline of this thesis

Despite extensive research on mammalian PI-TPs, progress in understanding their function has been slow during the last couple of years. The aim of this thesis is to obtain further insight in how these proteins regulate cell function. In **chapter 2** we report on the role of PI-TPα in the regulation of cell growth and survival. Activation of a PLA with affinity for PI by PI-TPα leads to the production of a COX-2-dependent anti-apoptotic factor mediating cell survival by activation of a cannabinoid 1-like receptor. In **chapter 3** the pathways by which the PI-TPα-dependent anti-apoptotic factor exerts is protective capability are described. It is shown by Western blot analysis and real life imaging that the p42/p44 MAP kinase and Akt/PKB pathways are activated upon incubation with conditioned medium (CM) from SPIα cells, leading to subsequent activation of transcriptional factor NF-κB. Increased expression of PI-TPβ renders SPIβ cells more sensitive towards UV-induced apoptosis. In **chapter 4** we provide data on the inhibition of the p42/p44 MAP kinase pathway in SPIβ cells and the production of an antagonist blocking the anti-apoptotic activity present in CM from SPIβ cells. In **chapter 5** several characteristics of the anti-apoptotic factor(s) present in CM from SPIα cells are described. In addition, a class of lipids is identified which is consistently different between CM from SPIα cells and CM from NIH3T3 cells and produced in a COX-2-dependent manner. In **chapter 6** the results presented in this thesis are discussed and put into a general perspective.

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Chapter 2

Phosphatidylinositol transfer protein α regulates growth and apoptosis of NIH3T3 cells: involvement of a cannabinoid 1-like receptor

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Abstract

Mouse fibroblast cells overexpressing phosphatidylinositol transfer protein α [PI-TPα; sense PI-TPα (SPIα) cells] show a significantly increased rate of proliferation and an extreme resistance toward ultraviolet- or tumor necrosis factor-α-induced apoptosis. The conditioned medium (CM) from SPIα cells or the neutral lipid extract from CM stimulated the proliferation of quiescent wild-type NIH3T3 cells. CM was also highly effective in increasing resistance toward induced apoptosis in both wild-type cells and the highly apoptosis-sensitive SPIβ cells (i.e., wild-type cells overexpressing PI-TPβ). CM from SPIα cells grown in the presence of NS398, a specific cyclooxygenase-2 (COX-2) inhibitor, expressed a diminished mitogenic and anti-apoptotic activity. This strongly suggests that at least one of the bioactive factor(s) is an eicosanoid. In accordance, SPIα cells express enhanced levels of COX-1 and COX-2. The anti-apoptotic activity of CM from SPIα cells tested on SPIβ cells was inhibited by ~50% by pertussis toxin and suramin as well as by SR141716A (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-cichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride), a specific antagonist of the cannabinoid 1 receptor. These inhibitors had virtually no effect on the COX-2-independent anti-apoptotic activity of CM from SPIα cells. The latter results imply that PI-TPα mediates the production of a COX-2-dependent eicosanoid that activates a G protein-coupled receptor, most probably a cannabinoid 1-like receptor.

Introduction

Phosphatidylinositol transfer proteins (PI-TPs) belong to a family of highly conserved proteins that *in vitro* can catalyze the transfer of phosphatidylinositol (PI), phosphatidylcholine (PC) and sphingomyelin (SM) between membranes [1, 2]. In mammalian tissues at least two isoforms are identified: PI-TPα, which is localized in the nucleus and cytosol and PI-TPβ, which is associated with the Golgi system [3]. Possible cellular functions of these proteins have been obtained from experiments with permeabilized cells [4], reconstituted Golgi membrane systems [5] and cells in which the

expression of the proteins has been altered [6-8]. In permeabilized, cytosol-depleted cells, both isoforms restored GTPγS-stimulated protein secretion as well as phospholipase C-mediated inositol lipid signaling [9-11]. On the other hand, intact mouse fibroblast cells with increased expression of PI-TPα (SPIα cells) showed an enhanced phospholipase A (PLA)-mediated degradation of PI that was not observed in cells with an increased expression of PI-TPβ (SPIβ cells) [7, 8]. Convincing evidence for distinct cellular functions was obtained by genetic approaches. Murine embryonic stem cells deficient in PI-TPβ fail to develop, whereas the embryonic development of cells deficient in PI-TPα proceeds normally [12, 13]. In the latter case, the mice die within two weeks of birth.

The rate of proliferation of SPIα cells is significantly increased, with a cell cycle duration of 13 h as compared with 21 h for wild-type cells, indicating that PI-TPα may be involved in the production of a mitogenic factor [7]. The observation that in SPIα cells a PLA with affinity for PI is activated implies that, in addition to lysoPI and glyceroPI, a significant amount of arachidonic acid is produced because PI is highly enriched in this fatty acid [14]. Arachidonic acid is the main precursor in the synthesis of eicosanoids, including prostaglandins, leukotrienes, thromboxanes and prostacyclins. These arachidonic acid metabolites play important roles in many cellular processes, such as thrombosis [15], inflammation [16, 17], cell growth [18] and apoptosis [19]. Eicosanoids are synthesized in response to external stimuli in which the release of arachidonic acid from phospholipids by PLA_2 is the rate-limiting step [20]. In particular, the prostaglandins, the synthesis of which depends on cyclooxygenase-1 (COX-1) and COX-2, play key roles in cell growth and cell survival as well as in processes such as carcinogenesis and inflammation [21]. In general, COX-1 is expressed constitutively, whereas COX-2 can be induced by various physiological stimuli [22-24]. Furthermore, it has been suggested that an increase in arachidonic acid metabolism as mediated by COX-1 or COX-2 depends on proteins that coordinate the release of arachidonic acid from phospholipids [25].

In the present study, we show that PI-TPα is involved in the regulation of proliferation and of apoptosis sensitivity. We present evidence that this regulation occurs through the COX-2-dependent production and secretion of eicosanoid factor(s). One of

these factors most likely acts on the G protein-coupled cannabinoid 1 receptor thereby displaying both autocrine and paracrine activity.

Materials and Methods

Materials

Indomethacin (Sigma), NS398 (Cayman Chemical), ELISA kit (Cayman), COX-1/COX-2 antibody (Cayman), [^{3}H]thymidine (Amersham), [^{14}C]arachidonic acid (Amersham), 4',6-diamidino-2-phenyindole (DAPI) (Sigma), Silica gel 60 TLC plates (Merck), prostaglandin E_1 (PGE_1), PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGD_2, PGA_2 (Sigma), suramin (Sigma), and pertussis toxin (Sigma). The cannabinoid receptor antagonists SR141716A and SR144538 were a kind gift of Dr. G. van Zadelhoff (Section Bio-organic Chemistry, the Bijvoet Institute, Utrecht University).

Preparation of conditioned medium

Cell cultures (55 or 150 cm^2 dishes) were grown to 80-90% confluency. The medium was replaced by 5 or 13.5 ml of DMEM/Bic containing 0.1% BSA (DBB). This medium was left on the cells for 24 h. After removal, the medium was centrifuged (5 min at 1000 rpm) to remove floating cells. The supernatant is the conditioned medium (CM). Under standard conditions, quiescent cells were incubated with CM that was derived from an identical surface of cells (i.e. each 9.5 cm^2 well of a six-wells dish was incubated with the amount of CM that was conditioned for 24 h by 9.5 cm^2 of cells.

Cell culture and growth assays

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (NCS) and buffered with $NaHCO_3$ (44 mM) in a 7.5% CO_2 and humidified atmosphere at 37˚C. NIH3T3 mouse fibroblast cells overexpressing PI-TPα (SPIα cells) and PI-TPβ (SPIβ cells) were made as described previously [7, 8]. In this

study, we have used two different SPIα cell lines (SPI2 and SPI8), giving essentially identical results.

To determine growth rate, cells (1 x 10^4 per well) were seeded on 24-well plates. After 24 h, the medium was replaced by 1 ml DMEM/Bic containing 0.1% NCS. After 24-48 h, the medium was replaced by 250 µl of CM or 250 µl of DMEM/Bic/0.1% NCS supplied with extracts of the medium. The volume of medium that had been conditioned by 2 cm^2 of cells was added to each well of a 24-well (2 cm^2 each) plate. After 8 h, [^{3}H]thymidine (0.5 µCi/well in 50 µl of DMEM/Bic/0.1% NCS) was added to the cells. After 16 h (overnight), the medium was removed, the cells were washed four times with phosphate-buffered saline and 1 ml of methanol per well was added. The methanol was removed after 20 minutes and the cells were left to dry on air. A total of 0.5 ml of 0.1N NaOH was added and the cells were incubated for 30 minutes at 37°C and scraped of. The cells were mixed with 4.5 ml of scintillation liquid and counted. Controls were cells incubated with 250 µl of DMEM/Bic/0.1% NCS. Maximal stimulation was obtained by the addition of DMEM/Bic containing 10% NCS.

Ultraviolet radiation of cell cultures

For ultraviolet (UV) treatment, cells were grown in a 6- or 12-well dish. Before UV treatment, the cells were incubated for 4 h in bicarbonate-buffered DMEM containing 0.1% bovine serum albumin (DBB). All the compounds that were tested for activity were added to this DBB during the incubation. The medium was removed and UV treatment was performed in a Stratalinker (Stratagene) with the indicated dose (standard: 200 J/m^2). Cells were incubated overnight (19 h) with pertussis toxin (300 ng/ml) in DMEM containing 10% newborn calf serum followed by incubation of pertussis toxin in CM from SPIα cells for 4 h; suramin and the cannabinoid 1 and 2 receptor antagonists, SR141716A (1 µM) and SR144538 (1 µM) were incubated for 4 h in CM from SPIα cells. After treatment with UV light, 1.5 ml of DBB was added and cells were incubated at 37°C. At the indicated times, cell death was morphologically scored as the percentage of cells that are in the process of blebbing, which have a condensed nucleus. Also, for visualization of condensed nuclei, the cells were grown on glass cover slips and similarly

treated. The cells were fixed in cold methanol (-20°C) and subsequently incubated with 1 μg/ml DAPI in methanol for 5 min at room temperature. Cells were washed once with methanol and once with PBS and mounted in Mowiol (Hoechst, Frankfurt, FRG) supplemented with 0.1% paraphenylene diamine. Fluorescent DNA-DAPI complexes were visualized in a Leica inverted microscope.

Flow cytometric analysis of DNA fragmentation

Cells were grown in 9 cm^2 dishes to 80% density. Cells were treated as described above (preincubation with conditioned medium for 4 h), UV treatment was performed and 1.5 ml of DBB was added to the cultures. Cells were kept at 37°C. After 2 h, the medium was collected and cultures were washed with 1 ml of PBS. This PBS was combined with the medium and centrifuged to collect floating cells. Cell cultures were incubated with 1 ml of 8 mM EGTA in PBS for 5 min, resuspended and centrifuged for 5 min at 800 rpm at 4°C. Both cell pellets were combined and resuspended in 100 μl of PBS supplemented with 0.5% polyvinyl alcohol (PVA), vortexed and kept on ice for 30 min. A total of 900 μl of 70% ethanol in PBS (0°C) was layered carefully on top of the PBS-PVA. The sample was mixed and stored at -20°C. Before flow cytometric analysis, nuclei were extracted and stained to detect nuclear fragmentation as sign of apoptosis (methodology with slight modifications is described by Li and Darzynkiewicz [26]). Briefly, the cell suspension was centrifuged for 3 min at 2200 rpm at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml of PBS and again centrifuged for 3 min at 2200 rpm. The pellet was resuspended in 1 ml of a mixture of 50% PBS and 50% extraction buffer (0.2 M Na_2HPO_4, 4 mM citric acid, pH 7.8). The suspension was kept for 10 min at room temperature. Nuclei were collected by centrifugation (3 min 2200 rpm), the pellet was resuspended in 1 ml of PBS containing propidium iodide (50 μg/ml) and RNase (50 μg/ml) and the suspension was incubated in the dark for at least 30 min. Before flow cytometric analysis, the mixture was filtered through a 70 μm cell strainer (Falcon) to remove clustered nuclei and other debris. Flow cytometric analysis of the extracted and stained nuclei (and fragments thereof) was performed on a FACScan dlow cytometer equipped with a 100 mW argon laser exciting

at 488 nm (Becton Dickenson, San Mateo, CA). The propidium iodide fluorescence of nonaggregated nuclear events was detected in fluorescence detector FL-3 (630 nm long-pass emission detector) in linear mode. Because it is likely that an apoptizing nucleus will fall apart into more than one event in the sub diploid area, the term apoptotic events will be used rather than apoptotic nuclei. This technique renders a relatively low scoring of apoptotic cells compared with visual scoring, as nuclear apoptotic events are still at an early stage at 2 h after UV irradiation.

Determination of COX-1 and COX-2

Cells were grown in 21 cm^2 dishes to 80-90% density. Cells were washed twice with PBS and conditioned medium was added. Cells were harvested after 5 h. To harvest the cells, the medium was removed, cells were washed twice with PBS and the dishes were frozen. After thawing, the cells were incubated with 150 μl of buffer containing 0.1% Nonidet P40 in 20mM Tris (pH 7.2) for 5 min at room temperature, scraped off and put on ice. The cell lysate was centrifuged for 10 min at 14,000 rpm at 4°C and the supernatant was used to determine protein content using the Bradford assay [27]. Equal amounts of protein of all samples were prepared for gel electrophoresis. A total of 20 μg of supernatant protein was subjected to SDS-PAGE on a 12.5% gel and analyzed by Western blotting using antibodies against COX-1 and COX-2. Quantification of bands on film was performed by scanning with a BioRad GS 700 imaging densitometer equipped with an integrating program.

Extraction of [^{14}C]-arachidonic acid-labeled metabolites from CM

Cells were grown to 60% confluency. The cells were labeled for 24 h with [^{14}C]arachidonic acid (0.1 μCi per well in a six-well plate in 1 ml of DMEM/Bic containing 10% NCS). To analyze labeled arachidonic acid metabolites in CM, the label medium was removed after 24 h and replaced by DBB. After 24 h, this medium was collected and centrifuged to remove floating cells. Arachidonic acid metabolites in the CM were extracted and separated as described by Tai et al [28]. Shortly, 0.03 ml of 12 M

formic acid was added per milliliter of CM. The mixture was extracted with two 3 ml portions of ethyl acetate. The combined extracts were evaporated under N_2. The residue was taken up in acetone and quantitatively spotted on a Silica Gel 60 TLC plate. The TLC plate was developed in the organic phase of ethyl acetate/acetic acid/iso-octane/water (11:2:5:10, v/v) as the solvent system. Radioactivity was monitored by scanning the plate with a Berthold Tracemaster 20 Automatic TLC-Linear analyzer.

Results

Mitogenic activity in CM from SPIα and wtNIH3T3 cells

Previously we have shown that SPIα cells have a cell cycle duration of 13 h compared with 21 h for wild-type NIH3T3 (wtNIH3T3) cells [7]. In view of this highly increased rate of proliferation, CM from SPIα cells was tested for the production of a mitogenic factor by measuring the incorporation of [^{3}H]thymidine into DNA of serum-starved (24-48 h) wtNIH3T3 cells. As shown in figure 1A, CM from SPIα cells increased DNA synthesis by a factor of 1.6. For comparison, CM from wtNIH3T3 cells increased the DNA synthesis by a factor of 1.1. This indicates that SPIα cells produce a mitogenic factor that is secreted into the medium.

Given the enhanced degradation of PI in SPIα cells as reflected in the relatively high levels of lysoPI and inositol phosphates [7], it is most likely that arachidonic acid also is released, which subsequently can be converted by COXs and lipoxygenases into arachidonic acid metabolites to be secreted into the medium [29]. To isolate prostaglandins, leukotrienes and other metabolites from CM, we used a neutral extraction procedure [28]. As shown in Figure 1B, the lipid extract of CM from SPIα cells increased DNA synthesis in quiescent wtNIH3T3 cells by 3.5-fold, whereas the extracts of CM from wtNIH3T3 had only a slight effect. By comparing fig. 1A and B, it appears that the mitogenic activity in the neutral lipid extracted is enhanced relative to that of CM. When the medium was conditioned in the continuous presence of 10 μM indomethacin, a nonselective inhibitor of COX-1 and COX-2, the CM was no longer mitogenic for quiescent wtNIH3T3 cells (Fig. 1C). In the presence of 50 μM NS398, a selective

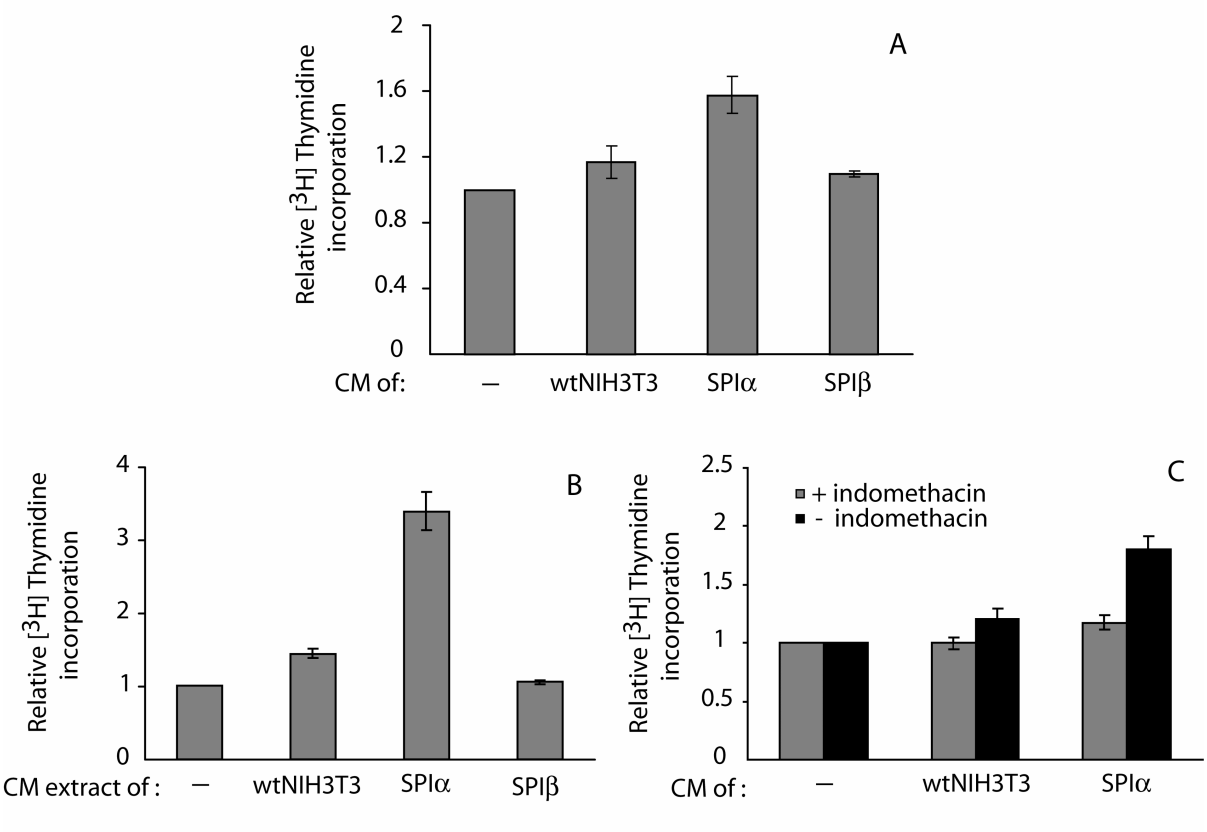

Figure 1. Mitogenic activity of conditioned medium (CM) from wild-type NIH3T3 (wtNIH3T3), SPIα and SPIβ cells tested on quiescent wtNIH3T3 cells. CM was prepared and the neutral lipid extract obtained as described in materials and methods. Mitogenic activity was determined by measuring the incorporation of [^{3}H]thymidin into DNA as described in materials and methods and presented relative to the control consisting of DMEM/bicarbonate containing 0.1% BSA (DBB). The activities of CM (A), of the neutral lipid extract (B), and of CM from wtNIH3T3 and SPIα cells prepared in the presence or absence of 10 μM indomethacin (C) are shown. CM prepared in the presence of 50 μM NS398 gave results identical to indomethacin. Results ± SD represent mean values of at least three experiments.

inhibitor of COX-2, the ensuing CM displayed a similar lack of mitogenic activity, indicating that the mitogenic factor(s) is dependent on COX-2 activity (data not shown).

The aqueous phase of the lipid extract was not active (data not shown), indicating that all mitogenic activity was present in the organic phase. Similarly, the neutral lipid extract from the postnuclear cell lysates was inactive, which indicates that all mitogenic activity formed was released in the medium.

Cell survival upon UV irradiation

Because the rate of proliferation is often correlated with apoptosis sensitivity [30, 31] we compared the apoptotic sensitivity of the wtNIH3T3 and SPIα cells upon UV irradiation. First, apoptosis was analyzed by quantifying the number of blebbing cells at 2 h after exposing the cells to variable doses of UV radiation (Fig 2A). Over a range from 20 to 400 J/m^2, the SPIα cells were almost completely resistant towards apoptosis, compared with wtNIH3T3 cells being up to 40% apoptotic. Apoptosis as a function of time upon a UV dose of 200 J/m^2 confirmed that SPIα cells are highly resistant towards UV-induced apoptosis (Fig 2B). Under the same conditions, the apoptosis sensitivity of cells overexpressing PI-TPβ (SPIβ) was also tested. Compared with the wtNIH3T3 cells, the SPIβ cells were found to be even more sensitive towards UV-induced apoptosis. This indicates that SPα cells have acquired a high resistance against apoptosis.

Factors that stimulate cell growth often also demonstrate anti-apoptotic activity [32, 33]. To explore the relationship between the mitogenic factor and apoptosis sensitivity, we investigated whether CM from SPIα cells is able to protect wtNIH3T3 cells against UV-induced apoptosis. Upon incubation for 4 h with CM from SPIα cells, the wtNIH3T3 cells were fully protected (Fig 2C). Similar protection was observed when wild-type cells were incubated with the neutral lipid extract of CM from SPIα. The CM from SPIα is also very effective in protecting the highly sensitive SPIβ cells [34] against UV-induced apoptosis (Fig 2D). Given that indomethacin inhibited the mitogenic activity of CM from SPIα cells (Fig 1C), we also determined the effect of indomethacin on the anti-apoptotic activity. As shown in fig 2D, CM collected in the presence of indomethacin (10 μM) was less effective in protecting SPIβ cells against UV-induced apoptosis. However, it is clear that the inhibitory effect of indomethacin on the mitogenic activity of CM from SPIα cells is more pronounced than its effect on the anti-apoptotic activity (cf. Fig 1C and 2D). This suggests that either the factor affects cell survival at a much lower concentration than cell growth or that the SPIα cells produce an additional anti-apoptotic factor independent of COX-1/COX-2.

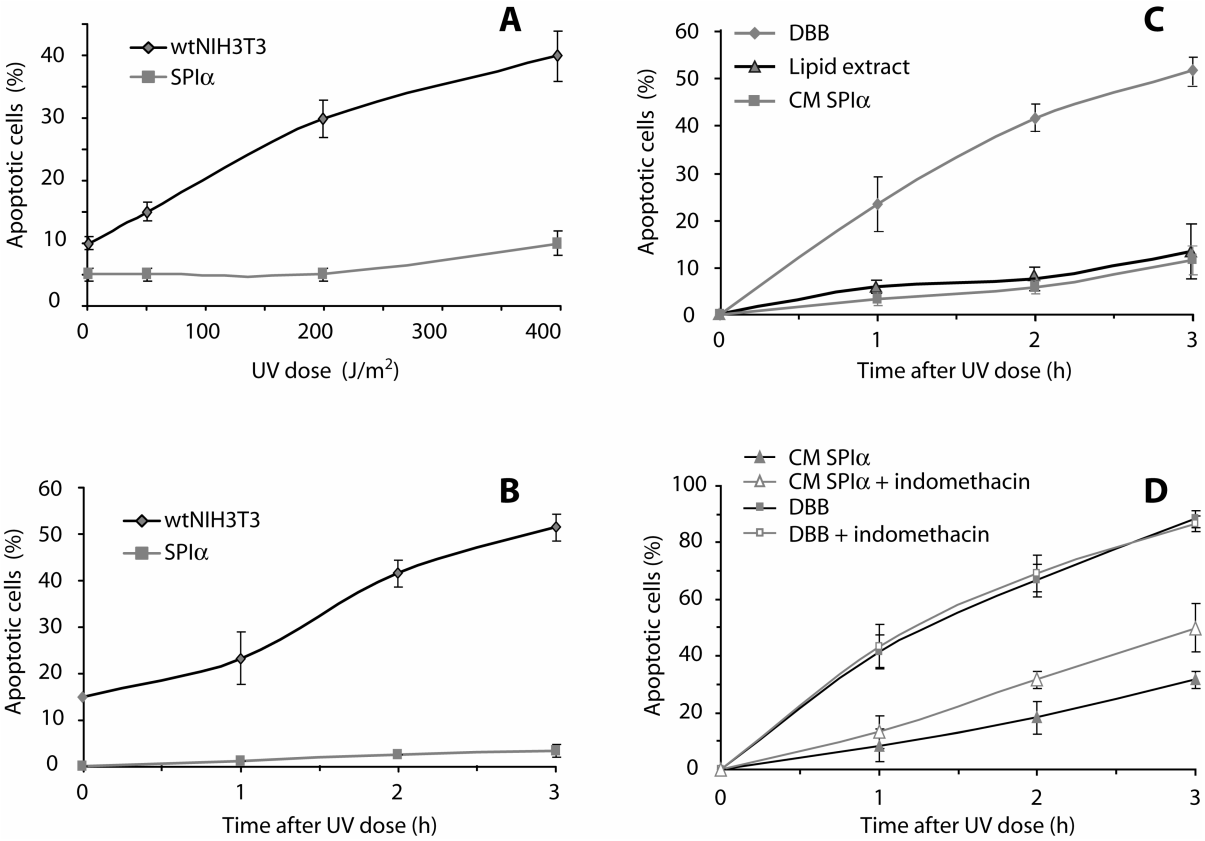

Figure 2. Survival of wtNIH3T3 and SPIα cells upon induction of apoptosis by ultraviolet (UV) radiation. Cells were grown to 90% confluency. The growth medium was replaced by DBB and the cells were incubated for 4 h at 37°C. After removal of DBB, the cells were irradiated as indicated, fresh DBB was added to the cells, and the number of apoptotic cells (blebbing) was counted at the indicated times. A: Percentage of apoptotic cells in wtNIH3T3 and SPIα cell cultures at 2 h after increasing doses of UV radiation. B: Time course of apoptosis in wtNIH3T3 and SPIα cell cultures after radiation with 200 J/m^2. C: wtNIH3T3 cells were incubated with DBB, with CM from SPIα cells, and with the neutral lipid extract thereof. At time 0, DBB and CM from SPIα cells were removed, cell cultures were radiated with 200 J/m^2 UV light, and fresh DBB was added. Percentages of apoptotic cells were determined at 0, 1, 2, and 3 h. D: SPIβ cells were incubated with DBB, with DBB containing indomethacin (50 μM), with CM from SPIα cells, or with CM from SPIα cells prepared in the presence of indomethacin (50 μM). At time 0, the media were removed, cell cultures were radiated with 200 J/m^2 UV light, and fresh DBB was added. Percentages of apoptotic cells were determined at 0, 1, 2, and 3 h. Results ± SD represent mean values of at least three experiments.

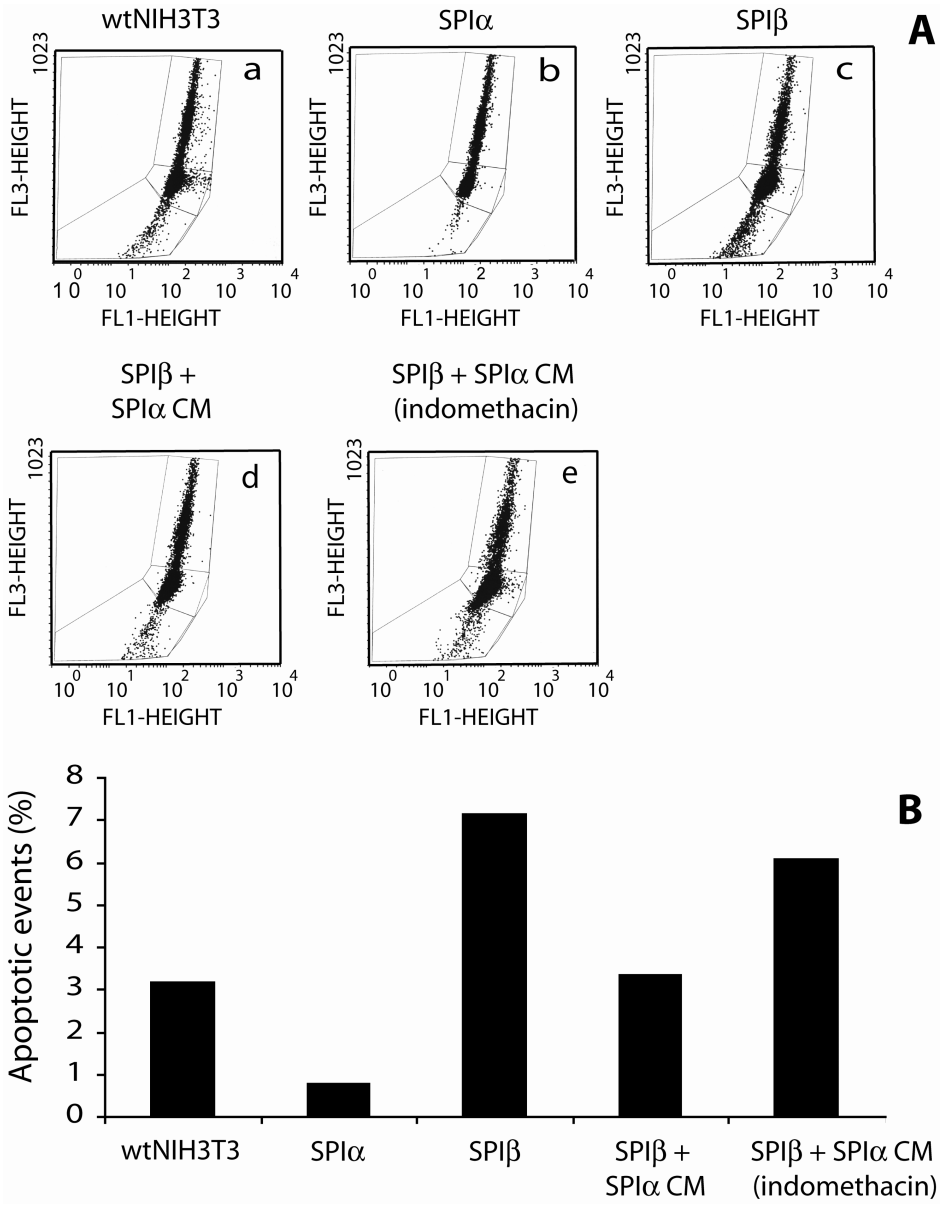

Figure 3. Survival of wtNIH3T3, SPIα, and SPIβ cells after UV irradiation as determined by flow cytometric analysis of the nuclei labeled by propidium iodide. Cells were collected at 2 h after UV irradiation (200 J/m^2) and prepared for flow cytometric analysis as described in materials and methods. A: Size-intensity diagram of the nuclei labeled with propidium iodide from wtNIH3T3 (a), SPIα (b), and SPIβ (c) cells incubated for 4 h with DBB before UV irradiation and from SPIβ cells incubated for 4 h with CM from SPIα cells prepared in the absence (d) or presence of 50 μM indomethacin (e). B: Percentage of apoptotic events representing the nuclei with reduced DNA (see area of A, bottom left). FL1, fluorescent signal from a photomultiplier tube with an excitation wavelength of 488 nm and an emission wavelength of 495–525 nm; FL2, fluorescent signal with an excitation wavelength of 488 nm and an emission wavelength of 630 nm.

In addition to counting blebbing cells, we quantified the percentage of apoptotic cells by analyzing DNA fragmentation by flow cytometric analysis of nuclei that were labeled with propidium iodide (Fig 3). Flow cytometric analysis carried out at 2 h after UV irradiation confirmed that the extent of apoptosis increased in the order: SPIα < wtNIH3T3 < SPIβ cells. When SPIβ cells were incubated for 4 h with CM from SPIα cells before UV irradiation, the percentage of apoptotic events was significantly decreased, confirming the anti-apoptotic activity of CM from SPIα cells. This protective effect was reduced using CM from SPIα cells prepared in the presence of indomethacin.

The different sensitivity of wtNIH3T3 and SPIα cells towards UV-induced apoptosis was confirmed by analysis of nuclear condensation using DAPI staining. Before serum starvation, the nuclear DNA of all wtNIH3T3 and SPIα cells was intact (Fig. 4A, D). After a sensitization by incubation for 4 h with DBB, the nuclei of some wtNIH3T3 cells showed DNA condensation, whereas the nuclei of SPIα cells were completely unaffected (Fig. 4B,E). This shows that in the case of wtNIH3T3 cells, serum deprivation by DBB is already an apoptotic signal. Two hours after UV irradiation, the nuclear DNA of the wtNIH3T3 cells showed a significant increase in condensation, whereas the effect on the nuclear DNA of SPIα cells was still minimal (Fig. 4C, F).

The mitogenic factor is an arachidonic acid metabolite

Indomethacin and NS398 block the production of the mitogenic activity, indicating that the bioactive factor in CM from SPIα cells is an arachidonic acid metabolite (Fig 1C). To obtain additional evidence, we prepared CM from SPIα cells labeled for 24 h with [^{14}C]arachidonic acid, extracted the medium with ethyl acetate and separated the lipid extract by TLC. As shown in Fig 5, four major [^{14}C]arachidonic acid metabolite peaks were present in CM from SPIα cells (right panel), whereas the bulk of ^{14}C label in CM from wtNIH3T3 cells ran with arachidonic acid (left panel). Similar analyses on CM from SPIα cells prepared in the presence of NS398 (50 μM), a specific COX-2 inhibitor, or of 10 μM indomethacin (data not shown) showed that three of the four metabolite peaks were absent. Using standards, it could be shown that PGE_2 and $PGF_{2\alpha}$ co-eluted

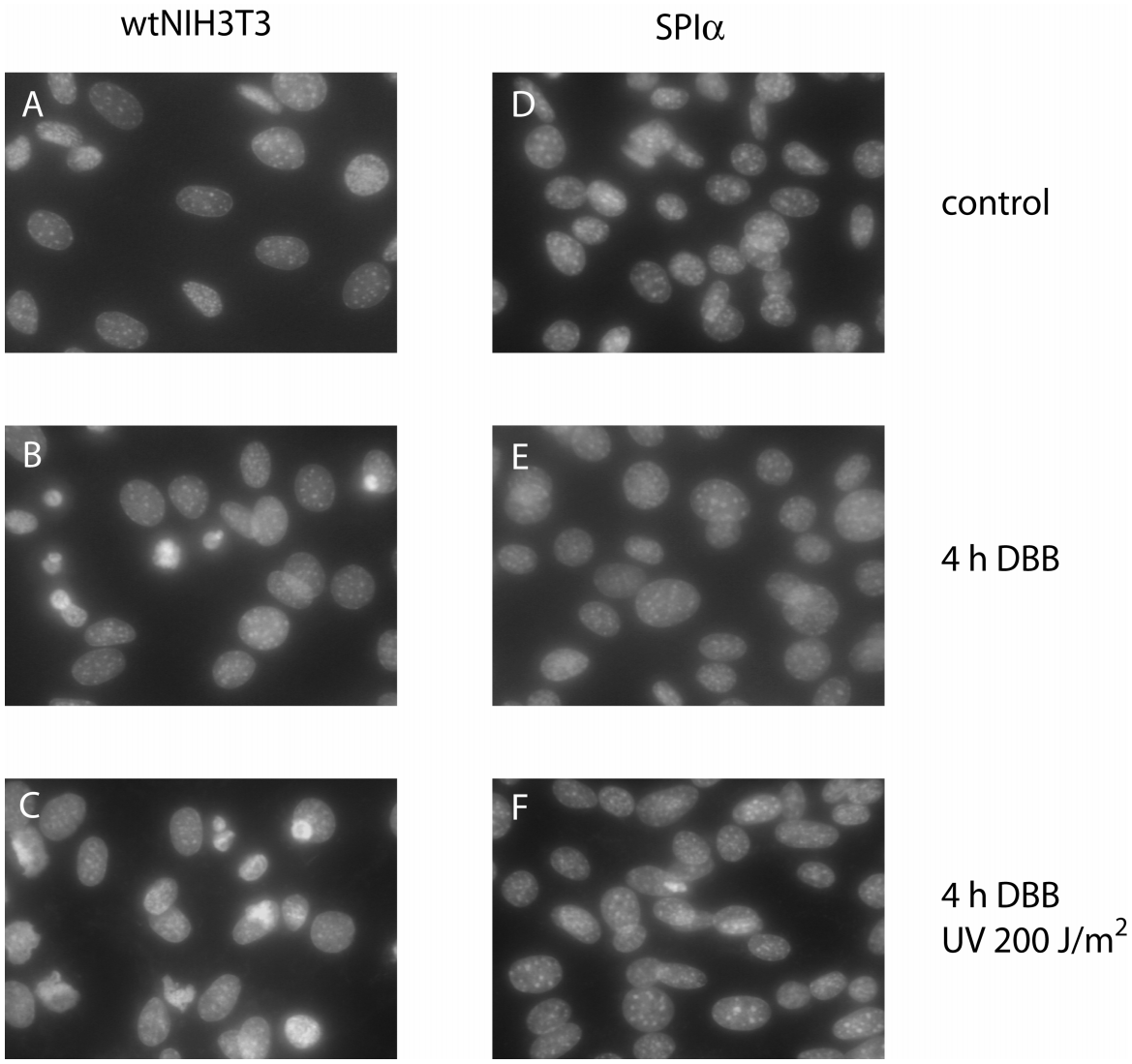

Figure 4. Survival of wtNIH3T3 and SPIα cells upon serum starvation and UV irradiation determined by 4',6-diamidino-2-phenyindole (DAPI) staining of condensed DNA. wtNIH3T3 cells (A–C) and SPIα cells (D–F) grown on glass cover slips were fixed and stained with DAPI as described in materials and methods. A and D: Control cells. B and E: Cells incubated with DBB for 4 h (serum starvation). C and F: Cells incubated for 4 h with DBB, irradiated with 200 J/m^2 UV, and fixed and stained after 1 h.

with two of the arachidonic metabolite peaks. By using an ELISA kit (Cayman) it was determined that SPIα cells produced five times more PGE_2 compared with wtNIH3T3 (0.5 ng/ml and 0.1 ng/ml, respectively). However, it was shown that both prostaglandins, either separate or combined, were unable to stimulate the growth of quiescent wtNIH3T3

cells in the concentration range present in CM from SPIα cells. Similar results were obtained by using PGE_1, PGD_2, PGA_2 and $PGF_{1\alpha}$ (data not shown), indicating that the COX-2-dependent mitogenic factor may be an as yet unidentified eicosanoid.

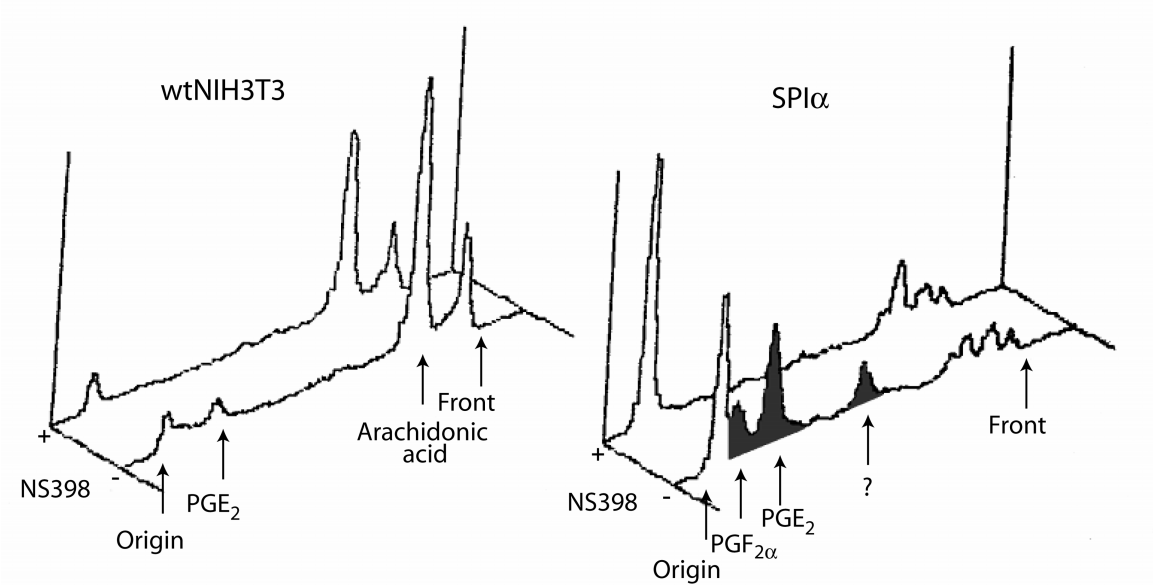

Figure 5. Thin layer chromatography scans of radioactive compounds in CM from wtNIH3T3 and SPIα cells prepared in the absence or presence of NS398 (50 µM). Cells were labeled to equilibrium with [^{14}C]arachidonic acid, CM was prepared and extracted by ethyl acetate, and the neutral lipid extract was separated and analyzed as described in materials and methods. Prostaglandins PGE_2 and $PGF_{2\alpha}$ and arachidonic acid were used as referents.

COX-1 and COX-2 expression

Given the high production of a mitogenic factor(s) by SPIα cells, we investigated the expression of COX-1 and COX-2 in these cells by Western blot analysis. In SPIα cells, the intensity of the immunoband of COX-1 and COX-2 are increased 4-fold and 7.5-fold, respectively, compared with wtNIH3T3 cells (Fig. 6). In addition, incubation with CM from SPIα cells for 5 h gives rise to a 3-fold increase in the intensity of the COX-2 band in wtNIH3T3 and a 2-fold increase in SPIβ cells, in line with the increased resistance of these cells towards UV-induced apoptosis (Fig 2C, D). Under these conditions, CM from SPIα cells has little effect on COX-1.

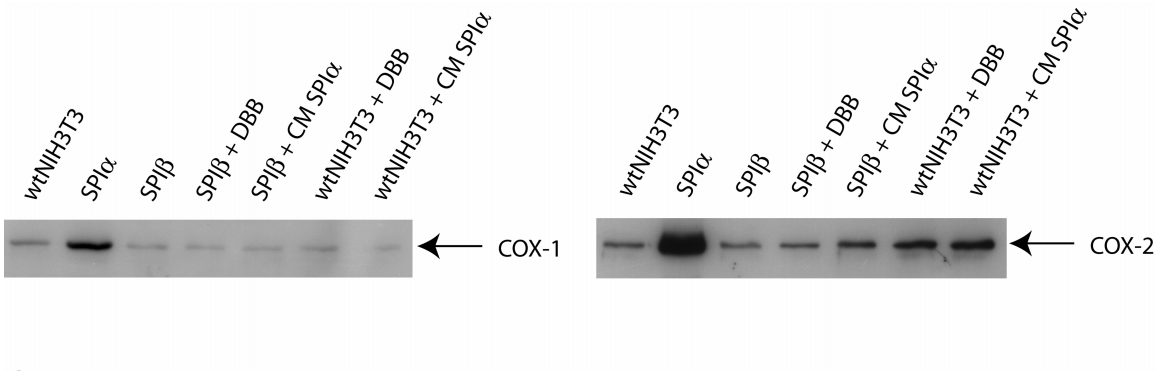

Figure 6. Western blot analysis of cyclooxygenase-1 (COX-1; left) and COX-2 (right) in wtNIH3T3, SPIα, and SPIβ cells and in wtNIH3T3 and SPIβ cells incubated for 4 h with DBB or CM from SPIα cells (SPIα CM). Cells were lysed, and the cytosolic fractions (aliquots of 25 μg of protein) were analyzed by SDS-PAGE and Western blotting using specific anti-COX-1 and anti-COX-2 antibodies as described in materials and methods. To ensure that identical amounts of protein were analyzed, gels and blots were routinely checked by Ponceau S/Coomassie Brilliant Blue staining.

Involvement of a G protein-coupled receptor

To identify the mode of action of the anti-apoptotic activity in CM from SPIα cells, inhibitors of G protein-coupled receptors (GPCR) were used. Incubation of SPIβ cells with CM from SPIα cells in the presence of suramin (300 μM) or pertussis toxin (300 ng/ml) inhibited the anti-apoptotic effect by ~40-50% (Fig. 7). A specific group of eicosanoids, the endocannabinoids, has been shown to inhibit neurodegeneration in rat brain by activation of a GPCR, the cannabinoid 1 (CB1) receptor [35, 36]. Because PI-TPα appears to play an important role in maintaining neural integrity [37], we investigated whether there was a relationship between the paracrine stimulation of SPIβ cells by CM from SPIα cells and the CB1 and CB2 receptors. Incubation with the CB1 receptor antagonist SR141716A (1 μM) for 4 h reduced the anti-apoptotic activity of CM from SPIα cells by 40 percent (Fig. 7). Under these conditions, the CB 2 receptor antagonist SR144538 (1 μM) appeared to have no effect. On the other hand, in contrast to pertussis toxin, suramin, and SR141716A, which had no effect on the apoptosis sensitivity of the SPIβ cells under control conditions (i.e., DBB), the CB2 receptor

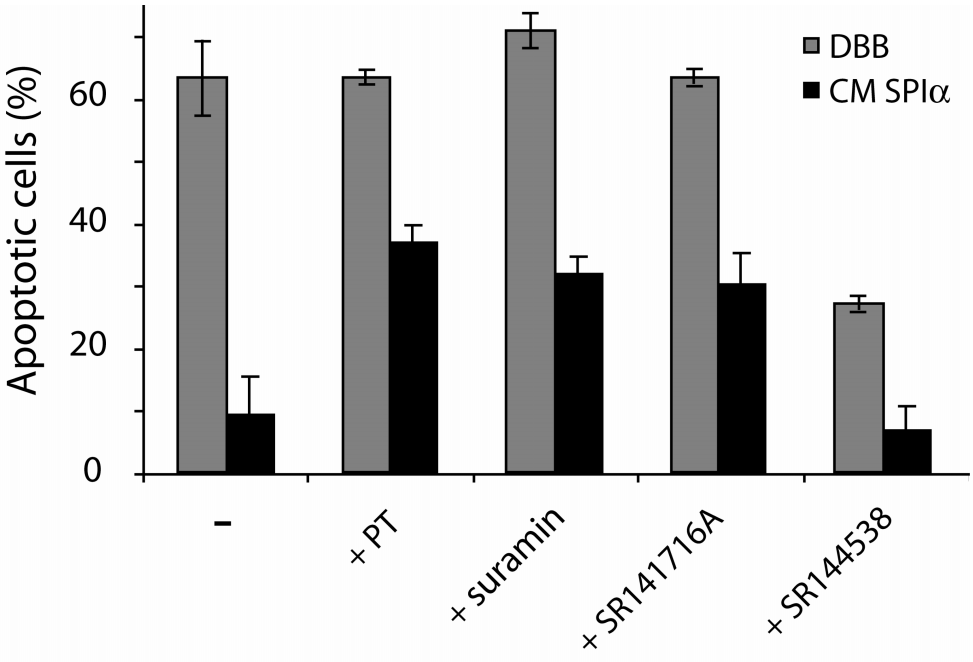

Figure 7. Protection of SPIβ cells by CM from SPIα cells in the presence of inhibitors of G protein-coupled receptors (GPCRs) and antagonists of cannabinoid 1 (CB1) or CB2 receptor. Cells were grown to 90% confluency. The growth medium was removed and the SPIβ cells were incubated for 4 h with DBB, with CM from SPIα cells, with CM from SPIα cells containing inhibitors of GPCRs [pertussis toxin (PT; 300 ng/ml) and suramin (300 μM)], and with CM from SPIα cells containing an antagonist of the CB1 receptor (SR141716A; 1 μM) or the CB2 receptor (SR144538; 1 μM). Gray bars represent data with DBB; black bars represent data with CM from SPIα cells. At time 0, the media were removed, cell cultures were irradiated with 200 J/m^2 UV light, and fresh DBB was added. Percentages of apoptotic cells were determined by visual scoring (blebbing cells) at 3 h. Results ± SD represent the mean values of four experiments.

antagonist SR144538 itself showed a distinct anti-apoptotic activity (Fig. 7). Hence, we cannot exclude the possibility that the anti-apoptotic factor also acts through the activation of the CB2 receptor. Moreover, addition of pertussis toxin, suramin or SR141716A to CM from SPIα cells prepared in the presence of NS398 failed to result in a significant further decrease of anti-apoptotic activity (data not shown). These results strongly suggest that at least one of the anti-apoptotic factors produced by SPIα cells is a COX-2-dependent endocannabinoid, acting through the activation of a CB1-like receptor.

Discussion

In this paper we show that NIH3T3 cells overexpressing PI-TPα (SPIα cells) are almost completely resistant towards UV-induced apoptosis compared with wtNIH3T3 cells. When CM from SPIα cells was added to wtNIH3T3 or SPIβ cells, which are extremely sensitive to UV irradiation, these cells also became apoptosis resistant. In addition, CM from SPIα cells as well as the neutral lipid extract derived from CM stimulates the growth of quiescent wtNIH3T3 cells. Anti-apoptotic and mitogenic activity in the CM was significantly diminished when the SPIα cells were incubated with COX-2 inhibitors during preparation of the CM. In line with previous observations on SPIα cells being enriched in lysoPI [7], CM from SPIα cells is highly enriched in arachidonic acid metabolites (e.g. 0.5 ng/ml PGE_2 compared with 0.1 ng/ml for wtNIH3T3 cells). This enrichment reflects the constitutive activation of PLA_2 by PI-TPα, resulting in the degradation of PI into lysoPI and arachidonic acid, which is subsequently converted into eicosanoids, among them PGE_2 and $PGF_{2\alpha}$. When prepared in the presence of the COX-2 inhibitor NS398, some major arachidonic acid metabolites were absent from CM from SPIα cells, suggesting that these compounds constituted a major part of the mitogenic and anti-apoptotic activity (see Fig. 4).

PI-TPα is localized in the cytosol and in the nucleus [3, 38]. The relevance of the nuclear localization is not clear yet. However, the presence of an active PI metabolism in the nucleus is well documented [39]. Furthermore, nuclei of several mammalian cells have been shown to contain an active acylation-deacylation cycle involving PLA_2 activity (reviewed in [40]). Additionally, because mammalian nuclei also contain a significant level of arachidonyl-PI [41], it remains to be established whether the PI-TPα-mediated production of lysoPI and arachidonic acid occurs partially in the nucleus.

In many cell types, PGE_2 is the major COX-2-dependent product known to promote cell growth in an autocrine and paracrine manner [42]. The mitogenic effects of PGE_2 are mediated by activation of E-prostanoid receptors [43]. When PGE_2 alone or together with $PGF_{2\alpha}$ was tested on quiescent wtNIH3T3 cells, we failed to observe the growth-promoting effect. However, PGE_2 and $PGF_{2\alpha}$ enhanced to some extent the survival of

these cells after UV irradiation. On the other hand, incubation of SPIβ cells with these prostaglandins had no effect on the survival of the SPIβ cells, which remained extremely sensitive (data not shown). This strongly suggests that SPIα cells produce another COX-2-dependent mitogenic eicosanoid, different from PGE_2 or $PGF_{2\alpha}$, the identity of which remains to be established. Similarly, we assume that these metabolites are also active in protecting cells against induced apoptosis.

It is well established that the induction of COX-2 prevents apoptosis by generating anti-apoptotic prostaglandins as well as by removing the pro-apoptotic substrate arachidonic acid [42, 44, 45]. In the case of the SPIα cells, the arachidonic acid released by PI-TPα from PI is effectively removed by COX-2, the level of which is highly increased compared with wtNIH3T3 and SPIβ cells (see Fig. 6). Incubation of the SPIβ cells with CM from SPIα cells increased the level of COX-2 but not of COX-1. This could explain why wtNIH3T3 and SPIβ cells are protected against apoptosis by CM from SPIα cells. On the other hand, CM from SPIα cells prepared in the presence of COX-1/COX-2 inhibitor indomethacin still showed considerable anti-apoptotic activity (see Figs. 2D, 3). This indicates that either the factor affects cell survival at a much lower concentration than cell proliferation or that, in addition to producing COX-dependent factors, SPIα cells also produce COX-independent anti-apoptotic factors.

Further insight into the nature of the factor was provided by the inhibitory effect of both GPCR inhibitors and the CB1 and CB2 receptor antagonists on the anti-apoptotic activity of CM from SPIα cells [46, 47]. The 40-50% reduction in anti-apoptotic activity induced by pertussis toxin, suramin and the specific CB1 receptor-antagonist SR141716A strongly suggests that the anti-apoptotic factor produced by SPIα cells is an endocannabinoid, which acts via activation of the CB1 receptor (Fig. 7). CM from SPIα cells prepared in the absence or presence of COX-2 inhibitors (indomethacin or NS398) in combination with the GPCR/CB 1 inhibitors showed a similar anti-apoptotic activity (i.e. 60 % of the control). This indicates that the activation of CB1 receptor is solely due to the COX-2-dependent cannabinoid [48, 49]. On the other hand, to date there is no direct evidence for the presence of CB1 receptor in mouse fibroblasts. Hence, we cannot exclude the possibility that the antagonist SR141716A acts on a CB1-like receptor.

These findings demonstrate a possible link between the production of endocannabinoids and PI-TPα activity. Decreased expression of PI-TPα in rat brain is known to cause serious neurodegeneration [37], whereas the endocannabinoid, anandamide has been shown to act as an endogenous protective factor of the brain against acute neuronal damage [35]. Our results provide evidence that the cell survival (e.g. the prevention of neurodegeneration) by PI-TPα is linked to the PLA-dependent release of

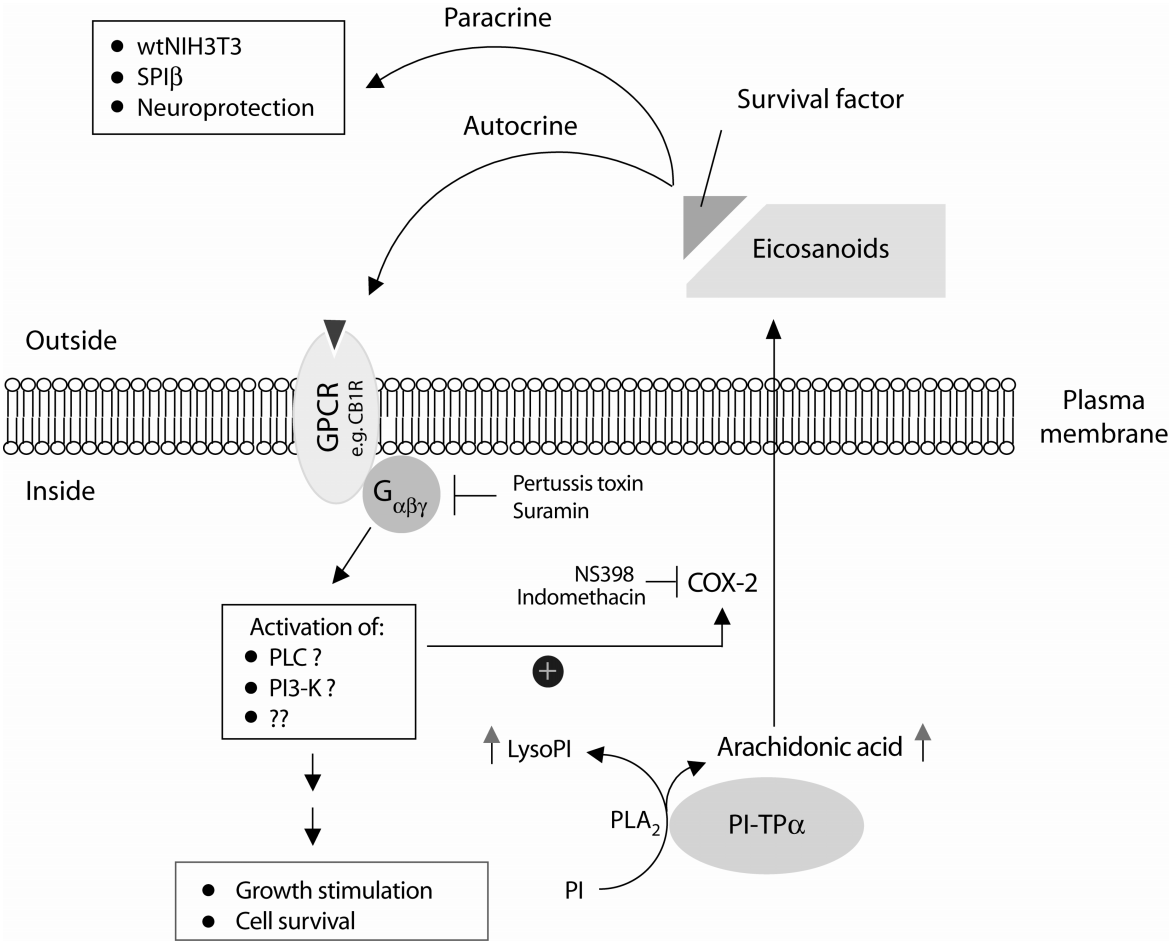

Figure 8. The regulatory role of phosphatidylinositol transfer protein α (PI-TPα) in the production of a bioactive eicosanoid. In the model presented, PI-TPα activates a phospholipase A (PLA) with affinity for PI leading to an increased release of arachidonic acid, which is subsequently converted into eicosanoids by COX-2. Part of these eicosanoids constitutes the survival factor(s), which has anti-apoptotic activity by acting through the activation of a GPCR, possibly cannabinoid 1-like receptor. PLC, phospholipase C; PI 3-K, phosphatidylinositol 3-kinase.

arachidonic acid, part of which is subsequently converted into bioactive eicosanoids by COX-2. The possible mechanism by which cell survival depends on PI-TPα is presented in a model (Fig. 8)

Recently mice lacking PI-TPα were shown to die within two weeks after birth as a result of massive physiological defects, including spinocerebellar degeneration, intestinal and hepatic steatosis and hypoglycemia [13]. Because *PI-TPα*$^{-/-}$ mice develop to term and are phenotypically normal, it appears that PI-TPα is not required for embryonic development. However, immediately after birth, these mutant mice failed to thrive demonstrating among other defects increasing apoptosis throughout the cerebellum. The importance of PI-TPα for normal brain function is also evident from the vibrator mouse, which have severe neurological disorders due to reduced PI-TPα levels in the brain [37]. Given that PI-TP transfer activity is particularly high in synaptosome and myelin fractions from rat brain as a result of high levels of PI-TPα [50], the neuronal cells of mice may be well protected against apoptosis by PI-TPα mediating the synthesis of the anti-apoptotic factor. Along this line, it will be interesting to investigate whether the early death of *PI-TPα*$^{-/-}$ mice after birth can be prevented by the administration (either in food or intravenously) of the eicosanoid factor produced by SPIα cells.

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Addendum

Apoptosis, the endocannabinoid - system, and the cyclooxygenase - system

Apoptosis

Cell death is a natural part of life, highlighted by the estimated number of three hundred million cells that die in the human body every minute. Cell death may occur in a disorganized, chaotic, and nonprogrammed manner, associated with swelling of the cell and inflammation of nearby tissue. This process is known as necrosis (referred to as Type III cell death) or, more specifically, oncosis [1]. Necrosis is typically characterized by swelling of the mitochondria, nuclear flocculation and uncontrolled cell lyses. Tissue necrosis is normally seen following severe trauma to cells or zonal killing following chemical toxicity [2].

A second mechanism of cell death is called programmed cell death, and involves the systematic disassembly of a cell without causing damage or stress to nearby cells. The concept of programmed cell death was first proposed by Lockshin and Williams (1964) proposing that cell death during development is an intentional procedure by which cells self-destruct according to a sequence of controlled steps [3]. To date two different forms of programmed cell death (PCD) are distinguished. The first type is autophagic (referred to as Type II cell death) cell death, characterized by the formation of large vacuoles that digest organelles in a specific sequence before the nucleus is destroyed [4]. The second type is called apoptosis (referred to as Type I cell death), which was first used by Kerr *et al*. [5]. The meaning of the Greek word απόπτόσισ is "falling off" in analogy of petals falling from a flower or of leaves from a tree in autumn, emphasizing that death is a vital ingredient in the life cycle of a multicellular organism.

The execution of apoptosis minimizes the leakage of cellular components from dying cells, thereby distinguishing from necrosis. Apoptotic cells can be characterized by a series of morphological changes: the cell shrinks, shows deformations and loses contact to its neighboring cells. Subsequently, the cytoplasm and nucleus condense, and the cell fragments into apoptotic bodies [6, 7]. The abundance of these morphological changes are a consequence of activation of a family of proteins known as caspases, which mediate the cleavage of DNA into oligonucleosomal fragments as well as the digestion of structural proteins, which determine the integrity and shape of the cells and its organelles [8].

Caspase activation as well as other biochemical and morphological changes are exploited in many techniques for measuring apoptosis. A number of more commonly used assays are the following:

Apoptosis is detected by visualizing the blebbing or budding of the plasma membrane through inspection of a cell under a light microscope.

Ordinarily many cells restrict phosphatidylserine (PS) to the inner leaflet of the plasma membrane by an energy dependent transporter. Redistribution of PS to the external surface is a feature utilized to detect apoptosis with annexin V coupled to a fluorescent conjugate. Fluorescence and, hence, apoptosis are visualized by flow cytrometric analysis or fluorescent microscopy.

DNA fragmentation is visualized by propidium iodide staining of isolated nuclear fragments followed by flow cytrometric analysis. Other techniques used are preferential DNA-end labeling and detection by the TUNEL assay and agarose gel electrophoresis showing DNA laddering.

A large number of biochemical changes can be detected by Western blot analysis using specific antibodies: cytochrome C release from mitochondria; cleavage of caspases or poly(ADP-ribose) polymerase (PARP); changes in bcl-2 inhibitory proteins [9, 10].

Many processes during development and maintenance of a multicellular organism depend on apoptosis [11].

Embryonic development

Apoptosis plays an important role during embryo development, metamorphosis and the downsizing of body organs (tissue atrophy). During embryonic development many

cells are produced in excess, which undergo PCD to shape fingers, toes and numerous hollow organs such as kidneys, lungs, blood vessels, eye lens and reproductive organs [11, 12]. In addition, PCD is critical in brain development, during which it is involved in axon guidance throughout the neuron proliferation phase [13, 14] [15, 16]. Moreover, within a differentiated neuronal population it is decisive for establishing definite patterns of neuronal connections, a process known as neurotrophic cell death. This process, by which more than 50% of all neurons undergo apoptosis, is regulated by the competition for a limited amount of nerve growth factor released by target cells [17-19].

Homeostasis

In a full-grown organism, there is continuous cell proliferation to renew tissue within various organs (e.g. hart, lung, liver, skin and blood). In view of the fact that the number of cells within a full-grown organ has to remain constant, the mitotic events have to be balanced by cell death [20]. This equilibrium is known as homeostasis and is regulated by both cell proliferation and apoptosis [21].

Damage, infection and immune system

Apoptosis is utilized to protect an organism against unwanted, damaged or dangerous cells. Cytotoxic T lymphocytes typically kill virus-infected cells by induction of apoptosis [22]. Moreover, native cells damaged beyond repair (e.g. DNA damage) are hazardous to an organism and must be removed. Upon extensive DNA damage the short-lived cellular stress response protein P53 is activated, leading to growth arrest and apoptosis [23]. Development and maintenance of the immune system is yet another field in which apoptosis has an essential role. The protection of mammals against infectious agents and virus-infected cells is primarily achieved by action of lymphocytes, more specifically B and T cells, which present functional antigen receptors on their plasma membrane. These antigen receptors are not tailored precisely to the antigen, but are generated through an erratic procedure. This results in a large variety of lymphocytes that are either ineffective, not recognizing the antigen or auto-reactive, recognizing self

antigens and attacking healthy native cells. The ineffective lymphocytes as well as the auto-reactive lymphocytes are removed by apoptosis. Furthermore, after an immune response the number of T lymphocytes is down regulated through apoptosis [24].

Apoptosis regulation and diseases

A family of proteases called caspases primarily facilitates the execution of apoptosis [25]. Caspases are synthesized in an inactive form called procaspases and are divided into initiator and effector caspases. In response to apoptosis signaling, the initiator caspases are cleaved and thereby activated. Subsequently, the active initiator caspases proteolytically activate the effector procaspases. Effector caspases then cleave the intended substrates in addition to procaspases themselves, resulting in the execution and magnification of the apoptosis signal [114].

Activation and inactivation of caspases are essential for regulation of apoptosis and are tightly controlled by proteins from the Bcl-2 family and by IAP's (Inhibitors of Apoptosis Protein) both regulated by the anti-apoptotic transcription factor NF-κB [26-28]. To date as much as 30 Bcl-2 family members have been identified in mammals consisting of both pro-survival member as well as pro-apoptotic members acting by the controlled release of cytochrome c from mitochondrion [29, 30]. The main pro-survival members are Bcl-2, Bcl-xl, Bcl-w, A1 and Mcl-1, all possessing the Bcl-2 homologue domains BH1 to BH4. The pro-apoptotic members can be divided in two groups, i.e. the Bax-subfamily containing BH1 to BH3 (e.g. Bax, Bak and Bok), and the BH3-only subfamily (e.g. Bik, Bad, Puma and Spike) [31, 32]. Both pro-survival and pro-apoptotic Bcl-2 members exert their effect on mitochondrial integrity. IAP's consist of c-IAP1, c-IAP2, XIAP and surviving which regulate apoptosis by direct interaction with caspases (reviewed in [33] and [34]).

Malfunctions in the regulatory system for apoptosis can lead to various diseases. Suppression of apoptosis results in cancer (homeostasis distortion) as well as in autoimmune disease. On the other hand, the increase in apoptosis could result in Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis by the induction of apoptosis in neurons, resulting in neurodegeneration. The

discussion whether neuronal death during neurodegeneration is necrotic or apoptotic is still continuing, although evidence is emerging that apoptosis is the main mechanism of cell death in the above mentioned diseases [35-39]. Furthermore, excessive apoptosis could contribute to AIDS (T cells committing suicide) [40, 41].

The endocannabinoid - system

Marijuana has been used for thousand of years for its fiber, oil and psychoactive properties by the Chinese and ancient Hindus [42, 43]. It was only in 1964 that the psychoactive compound from marijuana, Δ^9-tetrahydrocannabinol (THC) was isolated and characterized [44]. It was initially suggested that THC perturbed the plasma membrane so as to exhibit its cellular effect. This was shown to be incorrect when Matsudo *et al*. [45] identified and cloned a G protein-coupled receptor that binds THC, i.e. the cannabinoid 1 receptor (CB1 receptor). In 1993 Munro *et al*. showed the existence of another THC binding receptor, termed cannabinoid 2 receptor (CB2 receptor) [46]. Emerging evidence suggests the existence of a "CB3" receptor, which is not cloned to date [47, 48]. The CB receptors are coupled to Gi/o proteins and are shown to modulate adenylyl cyclase, ion channels and extracellular signal-regulated kinases [49, 50]. In addition emerging evidence shows that CB1 receptor is linked to the lipid second messenger ceramide.

Presence of the CB1 receptor is established in central nervous system areas which are associated with motor coordination, learning, memory and higher cognitive functions such as cerebral cortex, hippocampus, cerebellum and basal ganglia [51]. In addition CB1 receptor is also found in the female reproductive system [52]. The CB2 receptor is predominantly localized in cells belonging to the immune system [46, 53]. The fact that THC is not synthesized in mammals suggested the presence of endogenous ligands. To date three endogenous cannabinoids are well characterized, all derivatives of arachidonic acid. Two endocannabinoids bind to both CB1 and CB2 receptor, namely arachidonoylethanolamide (anandamide) [54] and 2-arachidonoyl glycerol (2AG) [55, 56]. The third endocannabinoid isolated from pork brain, 2-arachidonyl glyceryl ether (noladin ether) was found to be specific for CB1 [57]. Recently a fourth endocannabinoid

has been identified, virodhamide which was found to have a low affinity for the CB1 receptor [58-60] (figure 1).

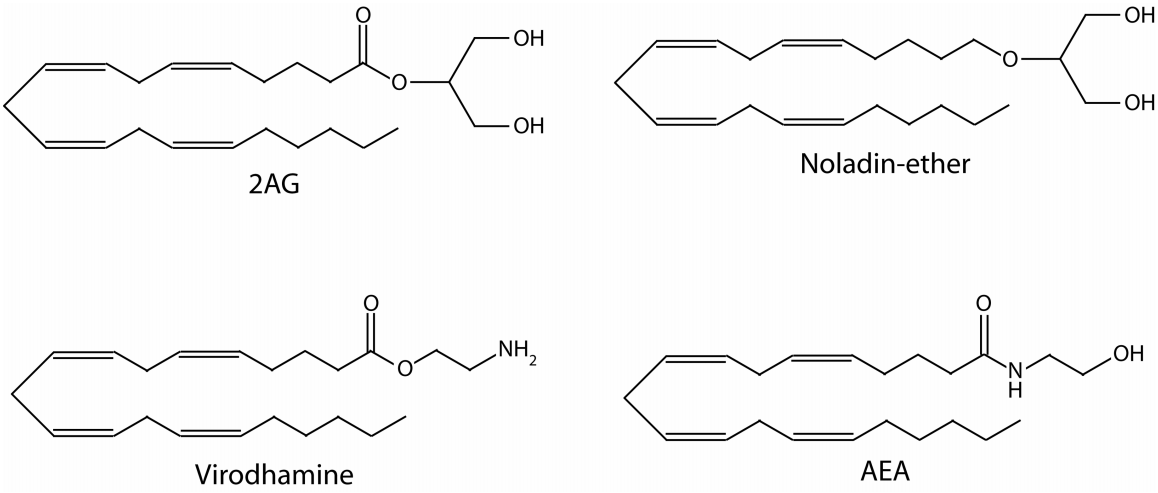

Figure 1. Four endogenous cannabinoids derived from arachidonic acid

The current model for anandamide (AEA) (figure 2) synthesis involves the transfer of the arachidonate moiety from the *sn*-1 position of 1,2-*sn*-di-arachidonoylphosphatidylcholine (arachidonoyl-CoA + 2-arachidonoyl lysophosphatidylcholine) to the primary amino group of phosphatidylethanolamide (PE) by an enzyme called N-acyltransferase (NAT) [61-63]. The lipid precursor N-arachidonoyl PE thus formed, is hydrolysed by PLD to result in the production of AEA [64-66]. Both synthesis of N-arachidonoyl PE and AEA are believed to be regulated by a rise in intracellular Ca^{2+} and/or by activation of neurotransmitter receptors [61, 67, 68]. Given that approximately 40% of the N-arachidonoyl PE is localized at the cell surface, AEA is most probably synthesized at the plasma membrane and released by passive diffusion or via lipid binding proteins such as lipocalins [69]. Cellular uptake of AEA is mediated by CB1 receptor, which in turn activates AEA membrane transports (AMT) [70, 71]. Once taken up by a cell, AEA is degraded into arachidonic acid and ethanolamide by fatty acid amide hydrolase (FAAH). FAAH is an intracellular membrane-bound protein of 64 kDa, which has been shown to degrade a broad range of

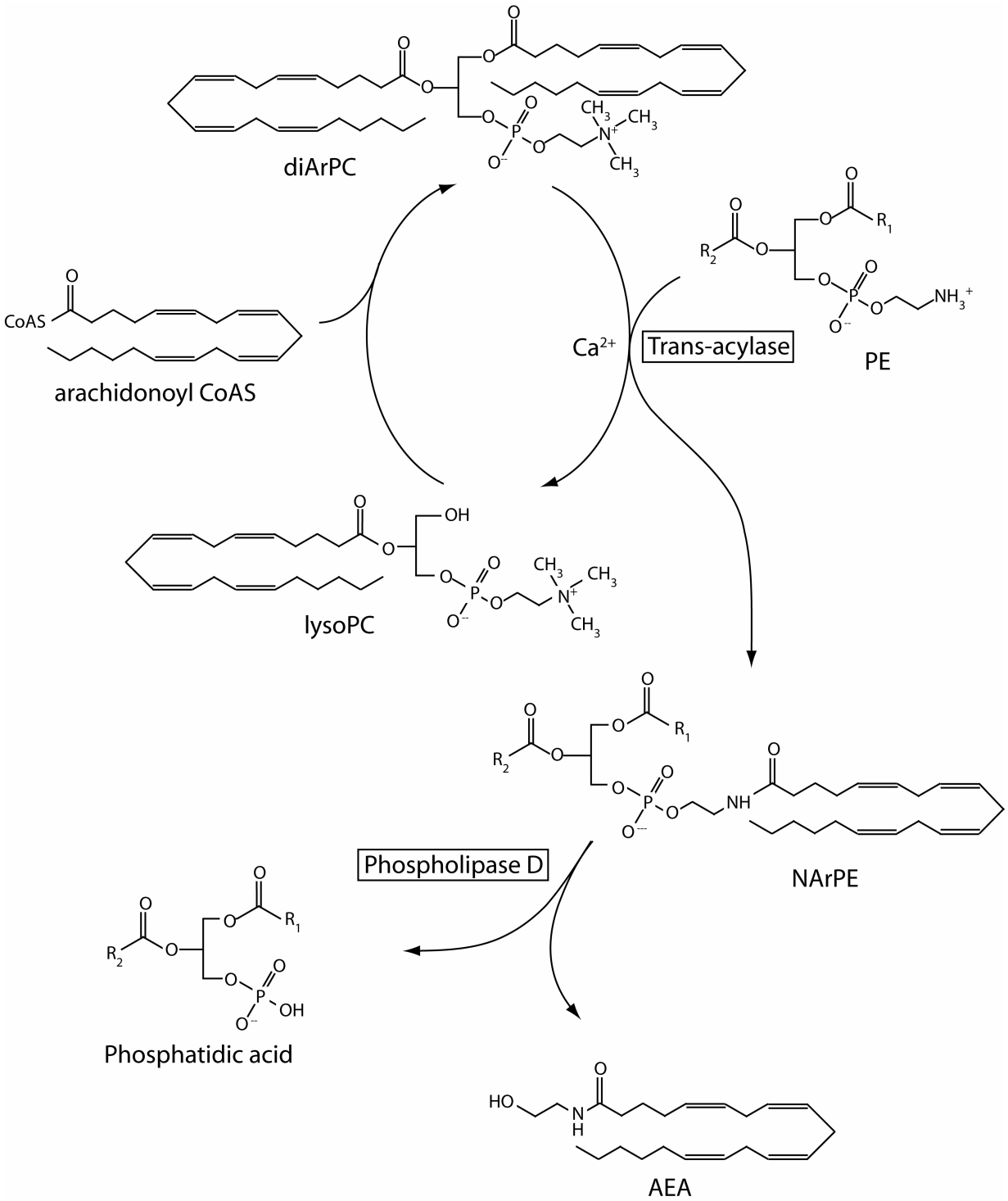

Figure 2. Biosynthesis pathway of anandamide. A calcium-dependent trans-acylase transfers the arachidonate moiety from the sn-1 position of 1,2-sn-diarachidonoylphosphatidylcholine (diArPC) to phosphatidylethanolamine (PE), thereby forming N-arachidonoyl-phosphatidylethanolamine (NArPE). The diArPC is regenerated by the reaction between the lysoPC and arachidonoyl-CoA. Hydrolysis of NArPE by a phospholipase D releases AEA and phosphatidic acid.

fatty acid amides and esters. Although cellular uptake of AEA is mediated by the CB1 receptor, it was shown that AEA demonstrates an additional non-receptor mediated effect [72].

To date, two possible pathways for 2AG synthesis are known (figure 3). The first route shows PI conversion into phosphatidylinositol 4,5-bisphosphate (PIP_2) via PI(4)P. PLC mediated hydrolysis of PIP_2 results in the formation of diacylglycerol (DAG), which may be subsequently converted to 2AG by diacylglycerol lipase (DGL) activity. The alternative route describes PLA_1 generating lysoPI from PI by cleaving off the sn-1 acyl chain. Then 2AG formation results from the hydrolysis of lysoPI by lysoPLC activity. Experiments involving specific inhibitor for PLC and DGL show that the PLC/DGL pathway is most probably the predominant route [73]. Similarly to AEA, the production of 2AG is increased by elevated intracellular Ca^{2+} levels. Moreover, the site of synthesis, the release into extracellular compartments, and the cellular uptake are thought to be similar [73-76]. FAAH was shown to be able to degrade 2AG, however emerging evidence suggest that the degradation of 2AG occurs predominantly via monoglyceride lipase (MGL) [77-80]. MGL is a cytosolic serine hydrolase that converts 1- and 2-monoglycerides to glycerol and the corresponding fatty acid, thus deactivating 2AG.
Research into the function of endocannabinoids has revealed that 2AG and AEA are involved in neurotransmission. In short, upon stimulation of neurons endocanabinoids are released from a postsynaptic neuron, where they act on CB1 receptor of the presynaptic neuron resulting in a reduced release of neurotransmitters such as glutamate, acethylcholine, noradrenaline, dopamine, 5-hydroxytryptamine, γ-aminoburyric acid (GABA) and aspartate [81]. In order to be deactivated they are subsequently removed from the synaptic cleft by the uptake into neuronal or glial cells.

Many experiments involving antagonists for CB1 and CB2 receptors (SR141716A and SR144528 respectively) and $CB1^{-/-}$ mice have implicated the cannabinoid system in numerous physiological events. In the central nervous system endocannabinoids are believed to regulate pain processing, motorfunctions, feeding appetite, body temperature, and cognitive function such as sleep and memory. Furthermore, outside the central nervous system, endocannabinoids are involved in regulation of blood pressure, secretion of pituitary and steroid hormones, embryo implantation, tumor growth and

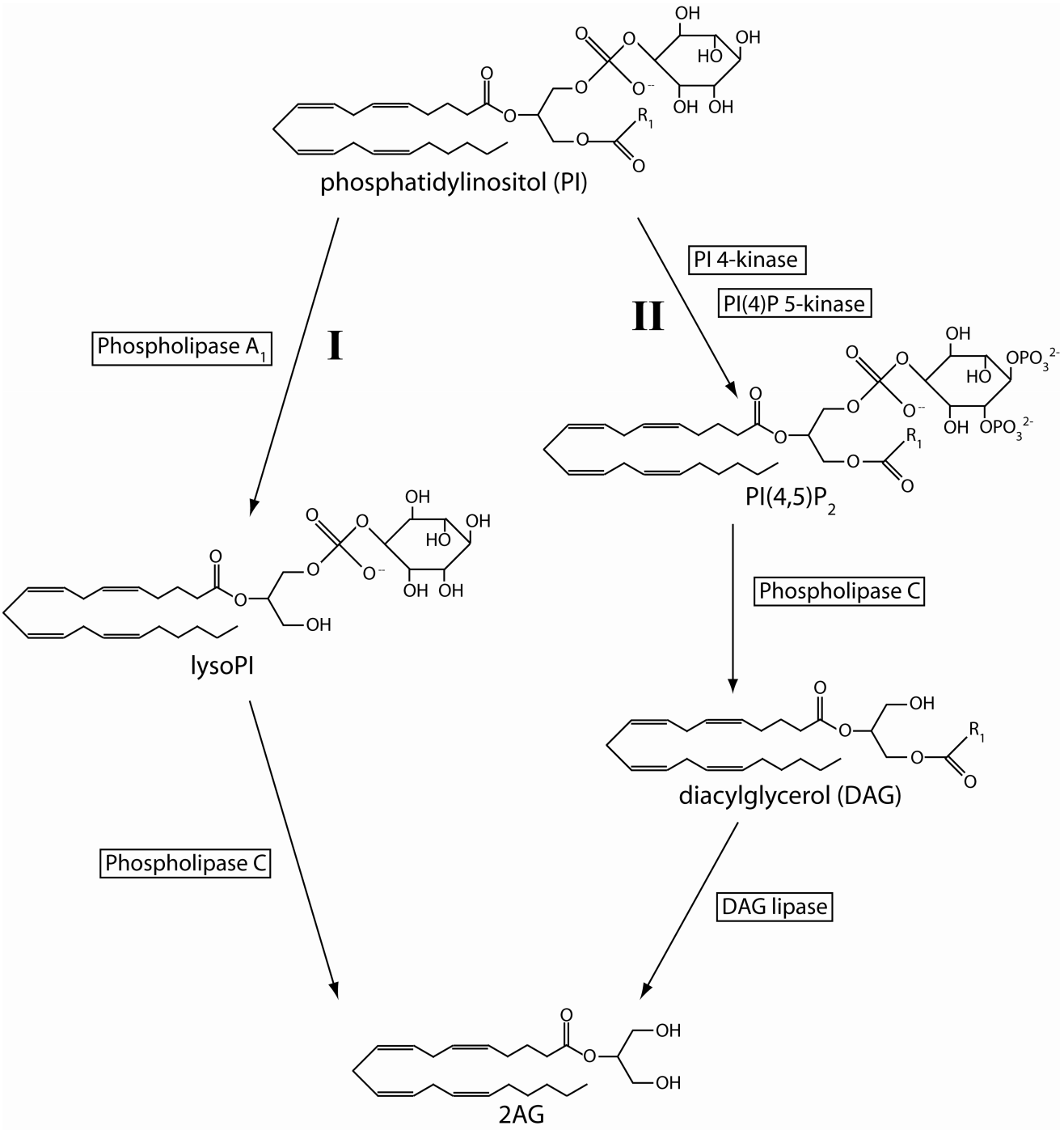

Figure 3. Biosynthesis pathways of 2-arachidonoylglycerol. I) Phospholipase A_1 cleaves off the acyl chain at the sn-1 position from phosphatidylinositol (PI). The hydrolysis of lysoPI by phospholipase C (PLC) results in the formation of 2-arachidonoylglycerol (2AG). II) The phosphorylation of PI by PI4-kinase and PI(4)P5-kinase results in the formation of $PI(4,5)P_2$. Diacylglycerol (DAG), formed by the hydrolysis of PI(4,5)P by PLC, is subsequently converted to 2AG by action of diacylglycerol lipase (DGL), which removes the acyl chain at the sn-1 position.

immunomodulation [52, 82-94]. Although the mechanisms by which cannabinoids influence neurodegeneration (i.e. Alzheimer's disease and multiple sclerosis) are still poorly understood, evidence shows a regulatory role for cannabinoids in the execution of programmed cell death. Cannabinoids like AEA and THC have demonstrated both a pro-apoptotic and an anti-apoptotic effect depending on the cell type utilized [95-98]. The pro-apoptotic action of AEA is attributed to the modulation of the balance between ARK, JNK and p38 MAP kinase by AEA [95]. On the other hand, the anti-apoptotic effect of cannabinoids is ascribed to the activation of the phosphatidylinositol 3-kinase/protein kinase B pathway, a well known fundamental anti-apoptotic signal [96-98].

The cyclooxygenase - system

Cyclooxygenases (COXs) catalyze the first two steps in the conversion of arachidonic acid (AA) to prostanoids, a large family of arachidonic acid metabolites, including prostaglandins (PGs), prostacyclin and thromboxanes. COXs insert molecular oxygen into AA to form the unstable intermediate PGG_2, which is rapidly converted to PGH_2 by the peroxidase activity of COX. The AA employed by these enzymes is derived from phospholipids or 2AG via action of PLC/PLA_2 or MGL respectively. To date, three isoforms of COX have been identified [99-102]. COX-1 is constitutively expressed in most tissues leading to relatively low levels of prostaglandins commonly believed to be involved in housekeeping functions [103]. The COX-2 isoform, an inducible enzyme, is regulated by several transcription factors including nuclear factor-κB (NF-κB), the nuclear factor for interleukin-6 expression (NF-IL-6) and the cyclic AMP response element binding protein [104]. In addition to being constitutively expressed in discrete population of neurons and enriched in the cortex and hippocampus, the expression of COX-2 is induced in a variety of cells by inflammatory and other stimuli, leading to high levels of prostaglandins [103, 105]. Initially it was demonstrated that COX-3 is constitutively expressed in canine cerebral cortex and to a less extent in other tissue. COX-3 is a smaller variant of COX-1 encoded by the same gene [102]. Since humans, rats and mouse lack the proper intron-1 in the mRNA encoding for COX-1, expression of COX-3 is not possible [106]. Localization studies show that all three enzymes are present

on the luminal surfaces of the ER and on the inner and outer membranes of the nuclear envelope [102, 107-109]. To date is has been established that the COX isoforms and its metabolites play important roles in a broad range of cellular responses like inflammation, gastrointestinal cytoprotection, angiogenesis, hemostasis and synaptic signaling by activation of G protein-coupled receptors such as the prostaglandin receptors E prostanoid 1 (EP1), EP2, EP3 or EP4. Furthermore, a role for COX has been implicated in various diseases like bone resorption, gastric ulceration, cancer, thrombosis, epilepsy, Alzheimer's disease and progression of kidney disease [106].

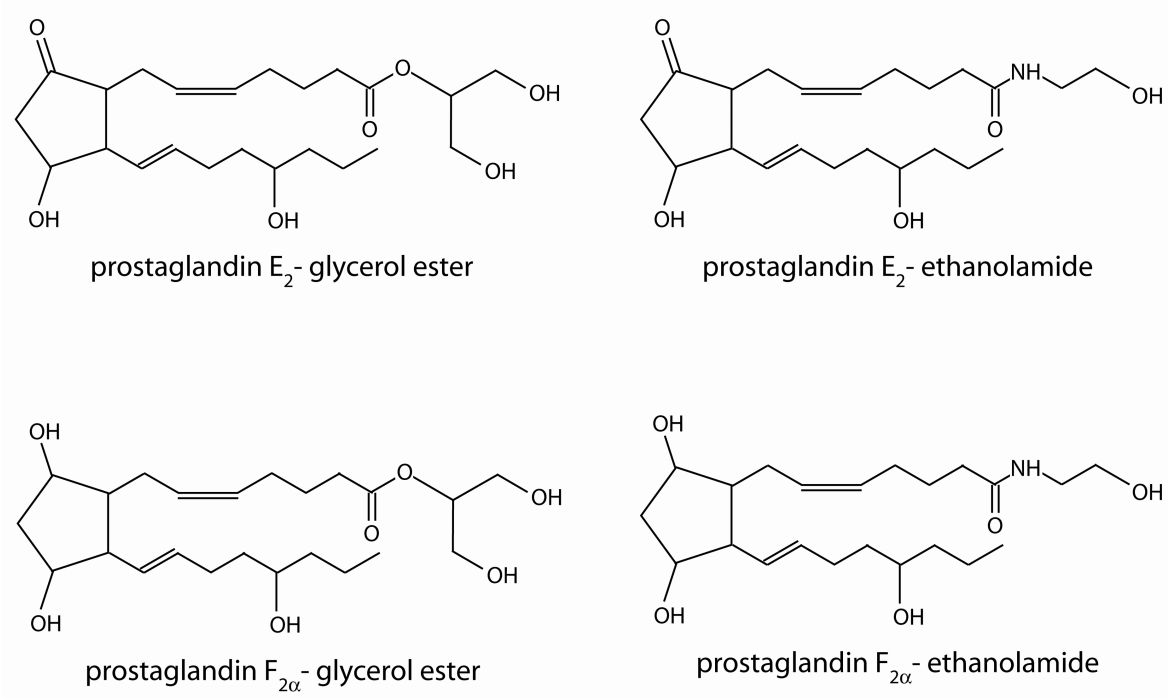

Figure 4. Cyclooxygenase-2-mediated (COX-2) formation of prostaglandin (PG) ethanolamides and PG glycerol ester. COX-2 can oxygenate endocannabinoids, thereby forming PG glycerol esters and PG ethanolamides, among which PGE_2 glycerol ester/ethanolamide and $PGF_{2\alpha}$ glycerol ester/ethanolamide.

Recently, a role for COX-2 in the cannabinoid system has been established. The synthetic cannabinoid WIN 55,212-2 increases the expression of COX-2, giving rise to an increased release of PGE_2 [110]. Furthermore, it was shown that in addition to converting AA to PG's, COX-2 is able to utilize AEA and 2AG as a substrate. To date, little is known about the function of these prostaglandin-like lipids formed by the COX-2-

mediated oxygenation of AEA and 2AG (figure 4). Emerging evidence shows that these metabolites called prostaglandin glycerol esters (PG-Gs) and prostaglandin ethanolamides (PG-Eas) have effects opposite to that of their precursors via a CB1 and prostanoid receptor-independent pathway [111-113].

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Chapter 3

The anti-apoptotic activity associated with phosphatidylinositol transfer protein α activates the MAPK and Akt/PKB pathway

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Abstract

The conditioned medium (CM) from mouse NIH3T3 fibroblast cells overexpressing phosphatidylinositol transfer protein α (PI-TPα; SPIα cells) demonstrates an increased anti-apoptotic activity compared with CM from wild-type NIH3T3 (wtNIH3T3) cells. This activity was evaluated using the apoptosis-sensitive SPIβ cells (i.e. wild-type cells overexpressing PI-TPβ) and acts by activating a G protein-coupled receptor, most probably a cannabinoid 1 (CB1) receptor [1]. The CB1 receptor is expressed in mouse fibroblast cells, at levels in the order SPIα>wtNIH3T3>SPIβ cells. Upon receptor activation, both the ERK/MAP kinase and the Akt/PKB pathway are activated as shown by Western blot analysis. Activation was also shown by EYFP-ERK2 translocation to the nucleus, as visualized by confocal laser scanning microscopy. The ensuing activation of the transcription factor NF-κB is in line with the increased resistance towards UV-induced apoptosis. On the other hand, receptor activation by CM from SPIα cells was not linked to phospholipase C activation as the YFP-labeled C2-domain of protein kinase C was not translocated to the plasma membrane of SPIβ cells.

Introduction

Phosphatidylinositol transfer protein α (PI-TPα) is a small, ubiquitously expressed protein belonging to a family of highly conserved proteins that in vitro transfers phosphatidylinositol (PI) and phosphatidylcholine (PC) between membranes [2, 3]. In mammalian tissues, two soluble, highly homologous isoforms are identified: PI-TPα, localized in the nucleus and cytosol and PI-TPβ, associated with the Golgi membrane [4-7]. Cellular functions of these proteins comprise the stimulation of secretory vesicle formation from isolated Golgi membranes, PI metabolism and inositol lipid signaling [8-11]. Furthermore, a decreased expression of PI-TPα and PI-TPβ was demonstrated in aged brain and in Parkinson's disease, linking PI-TP's to neurodegenerative diseases [12-14]. This was supported by the finding that PI-TPα$^{-/-}$ mice died within 14 days after birth as a result of spinocerebellar disease characteristics, hypoglycemia, and intestinal steatosis

[13]. Lethality of PI-TPβ gene ablation and difference in localization emphasizes that the PI-TP isoforms have separate physiological functions. A reduced expression of PI-TPα in rat WRK mammary tumor cells was reflected in a decreased rate of proliferation [9]. On the other hand, wtNIH3T3 mouse fibroblast cells expressing an increased level of PI-TPα (SPIα cells) have an enhanced rate of proliferation and are highly resistant towards UV- or tumor necrosis factor α-induced apoptosis [1, 10]. SPIα cells express a relatively high activity of PLA with affinity for PI, as shown by increased levels of lysoPI, inositol(1)phosphate, inositol(2)phosphate and glycerophosphoinositol. Furthermore, these cells secrete mitogenic and anti-apoptotic arachidonic metabolites, the production of which is partially dependent on cyclooxygenase- 2 [1]. The anti-apoptotic activity could be inhibited by inhibitors of G protein-coupled receptors and more specifically by a specific antagonist of the cannabinoid 1 (CB1) receptor [1].

Different signaling pathways linking surface receptor activation to cell survival have been described. Thus, it was shown that the p42/p44 mitogen-activated protein kinase (MAPK) pathway plays an important role in the growth and survival of eukaryotic organisms [15-19]. The MAP kinase pathway is stimulated upon binding of extracellular signals to both tyrosine kinase receptors, G protein-coupled receptors and cytokine receptors. Receptor activation leads to the subsequent phosphorylation of Ras, Raf, MEK (MAPKK), and extracellular signaling related kinase (ERK1/2) (reviewed in [20]). Upon activation of MEK, the MEK/ERK complex dissociates by which ERK1/2 is phosphorylated and translocated into the nucleus where it phosphorylates transcription factors [21-23]. It has been reported that ERK affects apoptosis by promoting expression of IAP's (inhibitor of apoptosis protein) [24-26].

Similar to p42/p44 MAPK, Akt/PKB has emerged as a key regulatory factor in several cellular functions such as cell growth, transcriptional regulation and cell survival [27]. Akt/PKB is a downstream effector of PI 3-kinase, which is activated by tyrosine kinase and G protein-coupled receptors [28]. Upon receptor activation, PI 3-kinase gives rise to an increased PI(3,4,5)P_3 formation. PIP_3 recruits Akt/PKB to the plasma membrane where it is activated by phosphorylation. In addition, it has been reported that Akt/PKB can be activated via protein kinase A [29, 30]. Upon activation, Akt/PKB has a direct effect on cell survival by inhibiting the pro-apoptotic Bcl-2 related protein, BAD and by inhibiting

caspases 9. Furthermore, Akt/PKB affects apoptosis on a transcriptional level both by activation of NF-κB and CREB, which regulate the transcription of pro-survival genes like Bcl-xL, caspases inhibitors and by inhibition of YAK and Forkhead, which regulate the transcription of pro-apoptotic genes like JNK and Bax [31-35].

Here we provide evidence that the PI-TPα-dependent anti-apoptotic factors act via both the p42/p44 MAP kinase pathway and the Akt/PKB pathway thereby activating the anti-apoptotic transcriptional factor NF-κB.

Materials and methods

Materials

Anti-P-MAPkinase, anti-P-Akt/PKB, anti-IκBα and anti-P-IκBα antibodies were obtained from Cell Signaling technology. DMEM and Lipofectamine 2000 were obtained from Invitrogen. Cannabinoid 1 receptor antibody was a kind gift of Maurice R. Elphick (School of Biological Sciences, Queen Mary and Westfield College, University of London). YFP-ERK2 was a kind gift of Andrey Shaw (Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, USA). C2-YFP was a kind gift of Tobias Meyer (Department of Molecular Pharmacology, Stanford University Medical Center, Stanford, USA).

Cell culture

All cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% newborn calf serum (NCS) and buffered with 44 mM $NaHCO_3$. Cells were maintained at 7.5% CO_2 at 37°C in a humidified atmosphere. wtNIH3T3 mouse fibroblast cells overexpressing PI-TPα (SPIα cells) and PI-TPβ (SPIβ cells) were made as described previously [10, 36].

Preparation of conditioned medium

Cells were grown to 90% confluency in 150 cm^2 dishes. After washing the cells twice with PBS, the medium was replaced with 13 ml of DMEM containing 0.1% bovine serum albumin (DBB medium). After 24 h the medium was collected and centrifuged (10 min at 1000 rpm) to remove floating cells. The supernatant is the conditioned medium (CM). Under standard conditions 90% confluent cells were incubated with CM derived from an identical surface of cells (i.e. 9.5 cm^2 of cells per well of a six-well dish was incubated with the amount of CM derived from 9.5 cm^2 of cells).

Induction of apoptosis by UV irradiation

Cells were seeded in 6-well plates and grown for 48 h until ca 90% confluency. Before UV treatment, the cells were incubated in DBB medium. To investigate the effects on the sensitivity to apoptosis, cells were incubated with CM for 4 h prior to UV irradiation. The medium was removed and the cells were given a UV dose (standard 200 J/m^2) using a Stratalinker (Stratagene). After UV irradiation, the cells were incubated with DBB at 37°C. At the indicated time points cell death was morphologically determined as the percentage of cells that are in the process of blebbing.

Sample preparation for Western blot analysis

Cells were grown in 21 cm^2 dishes to 80-90% confluency and incubated with CM for the indicated times. Cells were washed twice with PBS (ice-cold) and lysed in 20 mM Tris-HCl pH 7.5 containing 0.1% (v/v) NP_{40}, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1 mM aprotinin and 1 mM PMSF. Cell lysates were centrifuged at 17,500xg for 10 min at 4°C and the protein content of the supernatant fractions determined using the Bradford assay [37]. Equal amounts of supernatant proteins (50 μg) were subjected to SDS-PAGE on a 12% gel and Western blot analysis was performed using specific antibodies. Bands on the immunoblot were quantified using a Bio-Rad GS700 imaging densitometer equipped with an integrating program.

Fluorescence Microscopy

Cells were seeded in 6-well plates containing 24 mm Ø coverslips. The next day, cells were transfected with 1 μg purified plasmid DNA using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands). One day after transfection the coverslip was mounted in a home made chamber or an Attofluor cell chamber from Molecular Probes (Leiden, The Netherlands). Cells were observed and imaged on a LSM510 (Zeiss, Germany) confocal laser scanning microscope. A Zeiss 63x oil-immersion objective (Plan-Apochromat, NA 1.4) was used. YFP was excited using the 488 nm laser line, which was reflected onto the sample by a 488 nm dichroic mirror. YFP fluorescence was passed through a 505-550 nm bandpass filter and detected with a pinhole setting corresponding to 1 airy unit.

Data analysis

The average nuclear fluorescence (YFP-ERK2) or membrane/cytosolic fluorescence (C2-YFP) of a single cell was measured at every time-point by selecting a region of interest (ROI) in the right area using the Zeiss LSM510 software version 3.2. The initial value was normalized to 1. Graphs were prepared using Kaleidagraph 3.6 (Synergy software, Reading, PA).

Results

Anti-apoptotic activity of CM from SPIα and wtNIH3T3 cells

Previously, we have shown that SPIα cells produce and secrete a potent mitogenic and anti-apoptotic factor [1]. As shown in figure 1, SPIβ cells are fully protected against UV-induced apoptosis upon incubate for 4 h with CM from SPIα cells. For comparison, CM from wtNIH3T3 cells also expresses anti-apoptotic activity, yet to a lesser extent (Figure 1). This difference in anti-apoptotic activity reflects the level of PI-TPα in these cells.

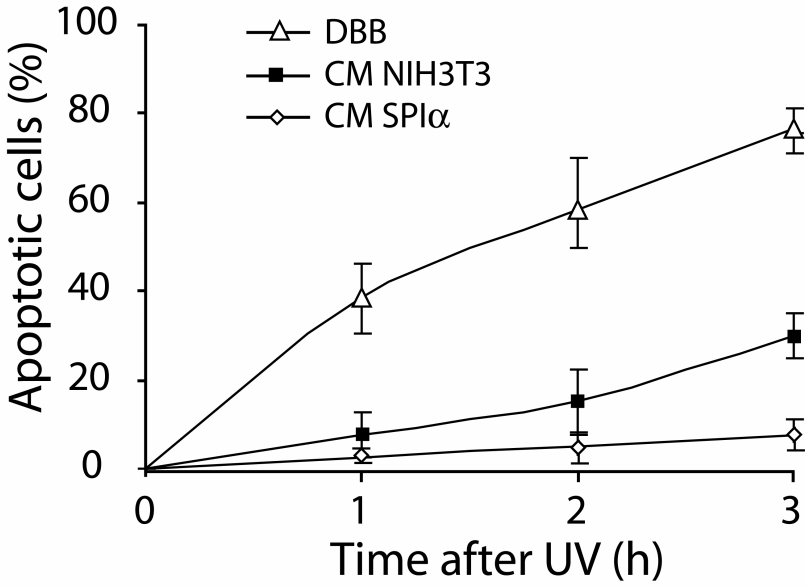

Figure 1. Survival of SPIβ cells upon induction of apoptosis by ultraviolet (UV) irradiation. SPIβ cells were grown to 90% confluency and incubated from 4 h at 37°C with DMEM/Bic/0.1% bovine serum albumin (DBB), CM from wtNIH3T3 or CM from SPIα cells. After removing the media, the cells were irradiated with UV light (200 J/m^2), fresh DBB was added to the cells and incubated for 3 h at 37°C. The number of apoptotic cells (blebbing) was determined by visual analysis at the indicated times. Results ± SD represent the mean values of at least three experiments.

CB1 receptor in wtNIH3T3, SPIα and SPIβ cells

Previously it was shown that the CB1 receptor antagonist SR141716A inhibited the anti-apoptotic activity of CM from SPIα cells [1]. By Western blot analysis using affinity-purified antibodies to the C-terminal 13 amino acids of the CB1 receptor, we show that the membrane fractions from wtNIH3T3, SPIα and SPIβ cells contain different levels of this receptor (figure 2). The immunoreactive 53 kDa band corresponds with the predicted molecular weight of the CB1 receptor, whereas the 62 kDa band corresponds with the glycosylated form of the CB1 receptor [38]. An additional immunoreactive band is detected at 45 kDa. The full length CB1 receptor appears to be prominently present in SPIα cells and to some extent in wtNIH3T3 cells. Moreover, the immunoreactive band at 45 kDa and the glycosylated CB1 receptor appear to be present to equal extents in wtNIH3T3 and SPIα cell and to a lesser extent in SPIβ cells. On the other hand, the effect

of the CB1 receptor antagonist and the protection by CM from SPIα cells indicate that the SPIβ cells have a functional level of the CB1 receptor.

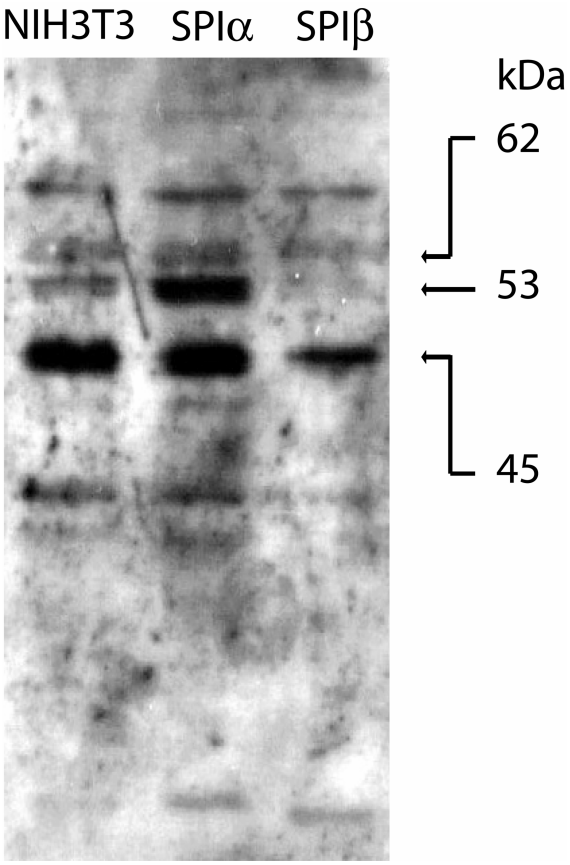

Figure 2. Expression of cannabinoid 1 receptor in NIH3T3, SPIα and SPIβ cells. Cells were grown to 90% confluency and membrane preparations were prepared as described in materials and methods. Equal amounts of membrane preparations (40µg) from wtNIH3T3, SPIβ and SPIβS262A cells were separated on an 8% polyacrylamide, Tris-glycine gel under nonreducing conditions. Gels were transferred to nitrocellulose and subjected to Western blot analysis using an affinity-purified cannabinoid 1 receptor antibody. To ensure that identical amounts of protein were analysed, blots were checked by ponceau S staining.

P42/P44 MAP kinase activation

Given that both CM from wtNIH3T3 and SPIα cells exhibited anti-apoptotic activity, we investigated whether the p42/p44 MAP kinase pathway was involved. Incubation of SPIβ cells with CM from SPIα cells gave rise to a rapid phosphorylation of p42/p44 MAP kinase over a time period of 30 min (Figure 3 A; left panel). After 1 h the level of phosphorylation returned to normal. For comparison, phosphorylation was much less upon incubation with CM from wtNIH3T3 cells (right panel).

Previously, it was shown that activation of ERK2 (p42 MAP kinase) is paralleled by translocation of a fluorescent-tagged ERK2 into the nucleus [39]. To visualize the

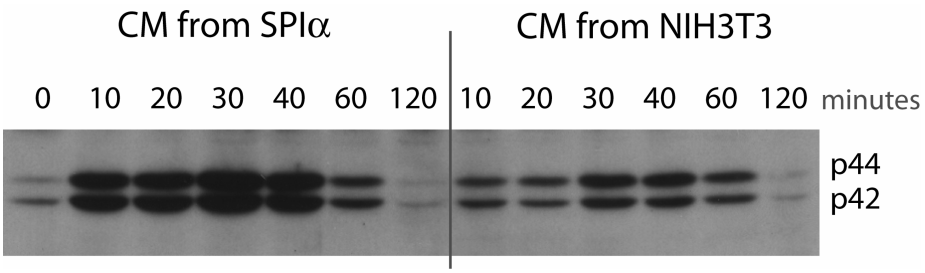

Figure 3A. Phosphorylation of p42/p44 MAPkinase in SPIβ cells upon incubation with CM from wtNIH3T3 or SPIα cells. SPIβ cells were grown to 90% confluency and incubated for the indicated times with CM from SPIα or wtNIH3T3 cells at 37°C. Equal amounts of cell lysate protein (25 μg) were subjected to SDS-PAGE followed by Western blot analysis using a p42/p44 MAPkinase specific antibody. Representative experiment performed in triplicate. Loading control was performed by ponceau S staining of the blot.

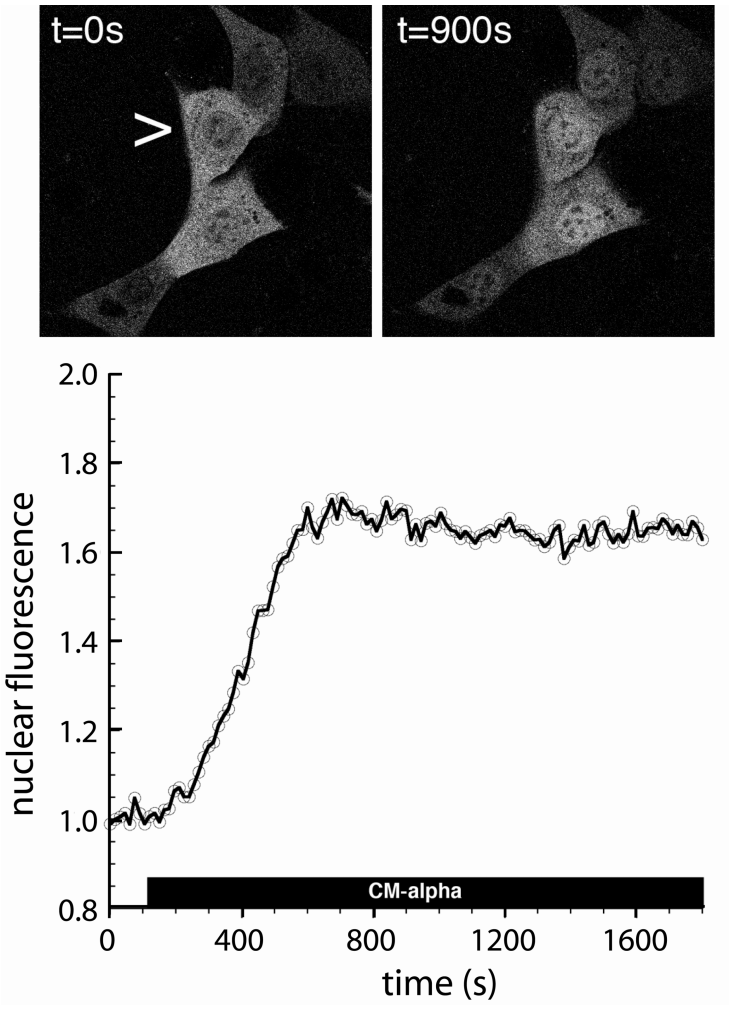

Figure 3B. Confocal images of SPIβ cells transfected with EYFP-ERK2 before (t=0s) and after stimulation with CM from SPIα (t=900s). The relative fluorescence intensity in the nucleus of the cell indicated with the arrowhead is quantified and shown in the graph (initial fluorescence is normalized to 1). The black bar indicates the presence of CM from SPIα cells. Width of a single image is 146 μm.

activation of the p42/p44 MAP kinase pathway with high spatial-temporal resolution in single living cells, SPIβ cells were transiently transfected with YFP-tagged ERK2 [40]. In resting cells, ERK2 is predominantly located in the cytoplasm. Upon addition of CM from SPIα cells, a clear accumulation of ERK2 in the nucleus was observed (Figure 3B). A rapid initiation of the ERK2 translocation was shown, attaining a maximum within 10 min (Figure 3B). These data agree very well with the rapid activation of the p42/p44 MAP kinase pathway observed by Western blot analysis.

Akt/PKB and NF-κB activation

To further establish the mode of action of the PI-TPα-dependent anti-apoptotic activity, the involvement of the Akt/PKB signaling pathway was studied by Western blotting using the antibody against Ser-473. Upon incubation of SPIβ cells with CM from SPIα cells, Akt/PKB was phosphorylated to a maximum extent at 10 min (Figure 4). Under these conditions, CM from wtNIH3T3 cells showed very limited Akt/PKB phosphorylation.

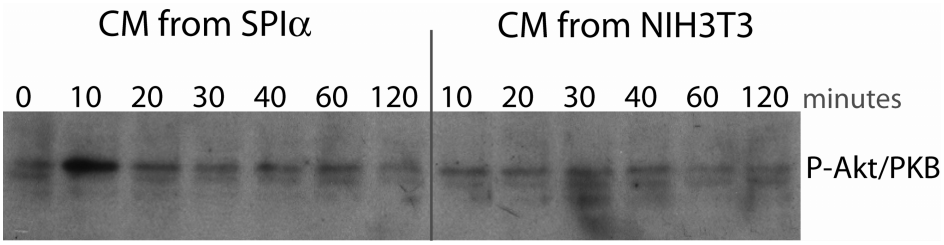

Figure 4. Phosphorylation of Akt/PKB in SPIβ cells upon incubation with CM from wtNIH3T3 or SPIα cells. SPIβ cells were grown to 90% confluency and incubated for the indicated times with CM from SPIα or wtNIH3T3 cells at 37°C. Equal amounts of cell lysate protein (50 μg) were subjected to SDS-PAGE followed by Western blot analysis using a P-Akt/PKB specific antibody. Representative experiment performed in triplicate. Loading control was performed by ponceau S staining of the blot.

Since the p42/p44 MAP kinase and the Akt/PKB pathways are activated, we also investigated the effect of CM from wtNIH3T3 and SPIα cells on NF-κB activation. NF-κB is retained in the cytoplasm in an inactive form by association with IκBα, the intracellular NF-κB inhibitor. Upon phosphorylation of IκBα by IKK (IκB kinase

complexes), the IκBα/NF-κB complex dissociates and p-IκBα is ubiquitinated and subsequently degraded in proteasomes. Both phosphorylation of IκBα at Ser-32 and the disappearance of IκBα from the cytosolic fraction indicate NF-κB activation. In agreement with the activation of the above pathways, incubation with CM from SPIα cells (10 min) gave rise to phosphorylation of IκBα (figure 5; lower left panel). Concomitantly, IκBα was cleared from the cytosol and reappeared again after 40 min (Figure 5; upper left panel). For comparison, incubation of SPIβ cells with CM from wtNIH3T3 cells showed less and a slower phosphorylation of IκBα and some clearance of IκBα from the cytosol (figure 5; right panels).

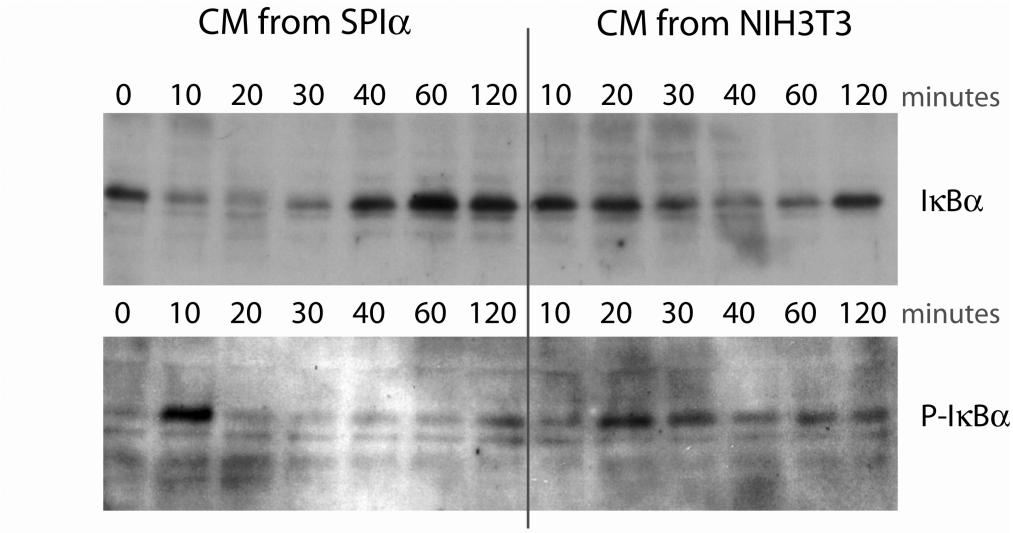

Figure 5. NF-κB activation in SPIβ cells upon incubation with CM from wtNIH3T3 or SPIα cells. SPIβ cells were grown to 90% confluency and incubated for the indicated times with CM from SPIα or wtNIH3T3 cells at 37°C. Equal amounts of cytosolic protein (25 μg) were subjected to SDS-PAGE followed by Western blot analysis using an IκBα or P-IκBα specific antibody visualizing clearance from the cytosol. Representative experiment performed in triplicate. Loading control was performed by ponceau S staining of the blot.

Phospholipase C activation

To investigate whether the PI-TPα-dependent survival factor could play a role in the activation of PLC, a calcium-sensitive C2 domain of protein kinase C fused to YFP was

used (C2-YFP). It has been demonstrated that C2-YFP is a sensitive reporter of PLC-mediated release of intracellular calcium [41, 42]. Upon calcium increase, the C2-YFP is translocated to the plasma membrane. SPIβ cells transiently transfected with C2-YFP displayed a homogeneous distribution of yellow fluorescence in the cytoplasm and nucleus (Figure 6A).

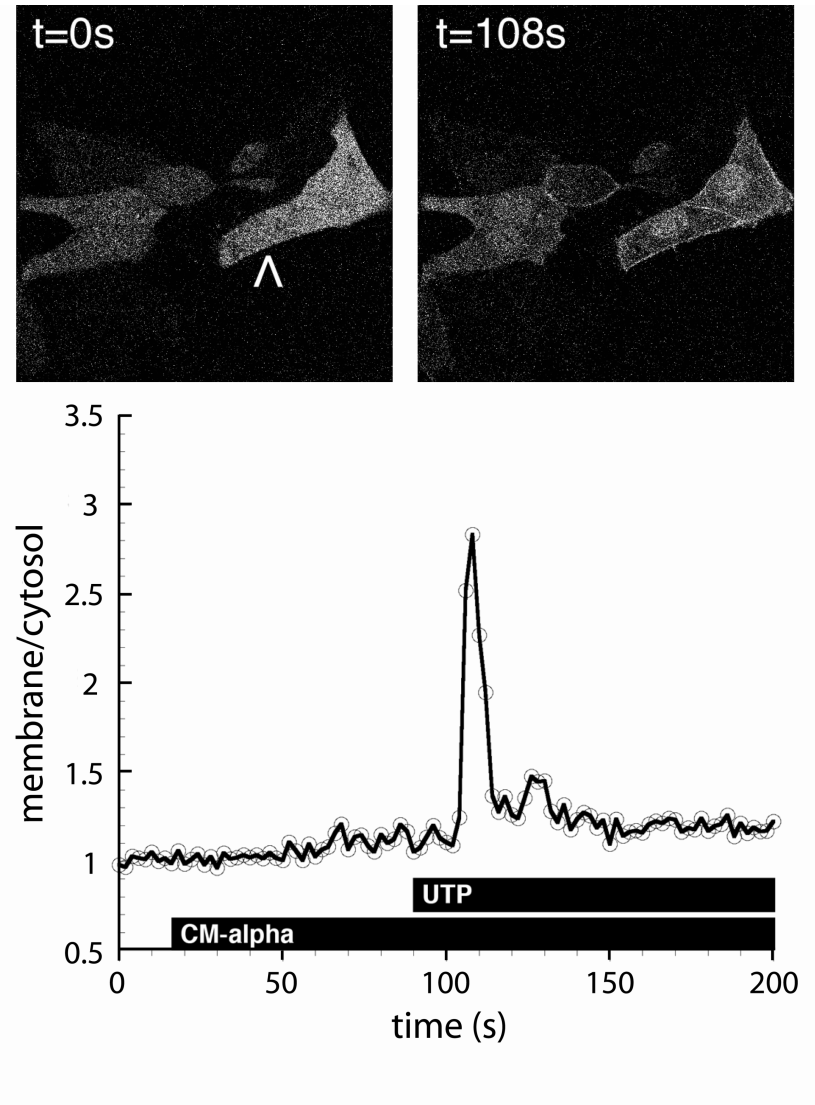

Figure 6. Confocal images of SPIβ cells transfected with the C2 of protein kinase C tagged with YFP before (t=0s) and just after stimulation with 100 µM UTP (t=108s). The fluorescence ratio of membrane/cytosol of the cell indicated with the arrowhead is quantified and shown in the graph (initial fluorescence is normalized to 1). The black bars indicate the presence of CM from SPIα and UTP in the medium. Width of a single image is 146 µm.

Upon addition of CM from SPIα cells the distribution remained unchanged. However, upon addition of uridine triphosphate (UTP; a potent PLC activator), the C2-YFP accumulated at the plasma membrane, indicating an increase in intracellular calcium (Figure 6A). Quantification of the ratio of membrane/cytosol fluorescence showed no change in the ratio due to CM from SPIα but a fast and transient increase in the ratio upon addition of UTP (Figure 6B). These data indicate that CM from SPIα does not activate PLC. This is in agreement with previous observations that the activation of the CB1 receptor is not linked to PLC activation [43].

Discussion

We have previously shown that wtNIH3T3 mouse fibroblast cells overexpressing PI-TPα (SPIα cells) produce a COX-2-dependent eicosanoid, which inhibits UV-induced apoptosis of SPIβ cells via activation of a G protein-coupled receptor (GPCR). Based on the inhibitory effect of the antagonist SR141716A, we propose that the GPCR is the cannabinoid 1 (CB1) receptor [1]. Here we report that wtNIH3T3 mouse fibroblast cells, SPIα and SPIβ cells express the CB1 receptor (53 kDa), although to different extents. Highest CB1 receptor levels were detected in SPIα cells, intermediate levels in the wild-type cells, whereas SPIβ cells exhibit a very low level of the CB1 receptor. An additional immunoreactive band is detected at 45 kDa (figure 1). Since there is evidence for alternative splicing of the human CB1 gene [44], the 45 kDa band may correspond to an isoform of the mouse CB1 gene. On the other hand, several studies report on the amino-terminal processing of the CB1 receptor [45, 46]. Due to the length of the N-terminal segment, the CB1 receptor cannot be efficiently translocated across the ER membrane. This leads to the degradation of the CB1 receptor by proteasomes and hence, to a low expression level at the plasma membrane. In accordance, it was shown in baby hamster kidney cells that a large number of the CB1 receptors are N-terminally truncated prior to ER translocation [46]. Similarly, the additional cross-reactive 45 kDa protein detected in mouse fibroblast cells may be a CB1 receptor which is N-terminally truncated. In addition, the observed change in the membrane-lipid composition of SPIβ cells (i.e. a

shift from short chain to long chain ceramide/SM species (Chapter 4)) may hamper membrane insertion of the CB1 receptor resulting in a reduced membrane expression of this receptor. In line with the low level of CB1 receptor in SPIβ cells and its high sensitivity towards UV-induced apoptosis, several studies report that invalidation or inhibition of this receptor enhances apoptosis [47-49]. On the other hand, CB1 receptor agonists like 2-arachidonylglycerol, arvanil and anandamide may inhibit as well as promote apoptosis [50-52]. In addition, studies on endocannabinoids converted by cyclooxygenases revealed that these metabolites have effects opposite to that of their precursors [53-55]. These findings emphasize the delicate role of the CB1 receptor and its agonists in the process of apoptosis.

The receptor expression runs parallel with the resistance against induced apoptosis. It may well be that an increase of the anti-apoptotic activity in CM upregulates the expression of the CB1 receptor (positive feedback). This would explain why in the case of the SPIβ cells where the anti-apoptotic activity is neutralized by an antagonist (Chapter 4), very low levels of CB1 receptor are observed. However, CM from SPIα cells is able to protect SPIβ cells in a CB1 receptor-mediated manner, indicating a functional level of CB1 receptor.

Cannabinoids exert most of their effects by binding the CB1 receptor at the plasma membrane thereby inhibiting adenylate cyclase (AC) and N- and P/Q-type voltage-sensitive calcium channels (VSCC), as well as activating mitogen- and stress-activated protein kinase (ERK, JNK, p38) and Akt/PKB pathways [15-17, 27, 56, 57]. In line with SPIα cells expressing 2-3 fold higher levels of PI-TPα compared with wtNIH3T3 cells, CM from SPIα cells displays a more pronounced effect on the survival of SPIβ cells than CM from wtNIH3T3 cells. An increase in the concentration of CM from wtNIH3T3 cells did not result in an anti-apoptotic effect comparable to CM from SPIα cells [unpublished data][49]. In addition, TLC analysis of neutral lipid extracts of CM from wtNIH3T3 and SPIα cells did not only show an increase in the concentration of arachidonic acid metabolites, but also a different composition [1]. This suggests that CM from SPIα cells contains additional anti-apoptotic factors which may be absent from CM from wtNIH3T3 cells. Comparison of both CM's revealed that the anti-apoptotic factors present in CM from SPIα cells are much more effective in the activation of the anti-apoptosis p42/p44

MAP kinase and Akt/PKB pathways and the subsequent activation of NF-κB. In addition, activation appeared to be rather specific as phosphorylation of p38 MAP kinase was not observed (data not shown). NF-κB activation upregulates the transcription of pro-survival genes encoding c-IAP1, c-IAP2, and IXAP, the TNF receptor–associated factors (TRAF1 and TRAF2) and members of the Bcl-2 family, in addition to upregulating a gene promoting cell proliferation, cyclin D1 a positive regulator of G1-to-S-phase progression [58-60]. In line with this, the anti-apoptotic activity present in CM from SPIα cells could exert its protective effect by the expression of all these factors. Since the activation of the Akt/PKB pathway also affects transcriptional factors like CREB, YAK and Forkhead, the activation or inhibition of other pathways affecting cells survival and cell proliferation is most likely.

Several studies show a relationship between PI-TPα and the GPCR-mediated activation of phospholipase C (PLC) upon addition of PI-TPα to cytosol depleted cells [11, 61]. By using SPIβ cells transfected with the plasmid containing cDNA encoding YFP-tagged C2 domain from PKC, we demonstrated that addition of CM from SPIα cells had no effect on the translocation of the fluorescent probe. Since the positive regulator of PLC (UTP) did result in translocation (Figure 6), it appears that the cellular effect of the anti-apoptotic factor is independent of PLC activation. This is in line with the fact that CB1 receptor signaling leads to the activation of the G protein $G_{i\alpha}$ which does not activate PLCβ [62-65]. This observation does not necessarily contradict the studies by Cockcroft *et al*. that PI-TPα acts directly in the cell through activation of PLC [66]. In the latter case, it is proposed that PI-TPα is essential for enhanced formation of PI(4)P and PI(4,5)P_2 as substrates of PLC.

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Chapter 4

The anti-apoptotic MAP kinase pathway is inhibited in NIH3T3 fibroblasts with increased expression of PI-TPβ

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Abstract

Mouse NIH3T3 fibroblast cells overexpressing phosphatidylinositol transfer protein β (PI-TPβ, SPIβ cells) demonstrate a low rate of proliferation and a high sensitivity towards UV-induced apoptosis compared with wild-type NIH3T3 (wtNIH3T3) cells. In contrast, SPIβS262A cells overexpressing a mutant PI-TPβ that lacks the protein kinase C-dependent phosphorylation site Ser-262, demonstrate a phenotype comparable to wtNIH3T3 cells. This suggests that the sensitivity towards apoptosis is related to the phosphorylation of Ser-262 in PI-TPβ.

Conditioned medium (CM) from wtNIH3T3 cells contains bioactive factors that are able to protect SPIβ cells against UV-induced apoptosis. CM from SPIβ cells lacks the protective activity. However, after heat denaturation CM from SPIβ cells regains a protective activity comparable to that of CM from wtNIH3T3 cells, which is not affected by heat-treatment. This indicates that CM from SPIβ cells contains an antagonistic factor interfering with the anti-apoptotic activity present. SPIβS262A cells do not produce the antagonist suggesting that phosphorylation of Ser-262 is required. Moreover, in line with the lack of anti-apoptotic activity CM from SPIβ cells does not induce the expression of COX-2 or the activation of p42/p44 MAP kinase in SPIβ cells. In contrast, CM from wtNIH3T3, SPIβS262A cells or heat-treated CM from SPIβ cells does induce these anti-apoptotic markers.

Since PI-TPα is involved in the production and secretion of a mitogenic and anti-apoptotic arachidonic acid metabolite(s), we investigated the effect of prostaglandin (PG) E_2 and $PGF_{2\alpha}$ on cell survival. These prostaglandins, which are prominently present in CM from SPIα, were found to protect wtNIH3T3 and SPIβS262A cells against UV-induced apoptosis but failed to rescue SPIβ cells. Concomitantly, upon incubation with PGE_2 and $PGF_{2\alpha}$, an increased expression of COX-2 and activation of p42/p44 MAP kinase were observed in wtNIH3T3 and SPIβS262A cells but not in SPIβ cells. Hence, it appears that specific mechanisms of cell survival are impaired in SPIβ cells.

In summary, the apoptosis sensitivity of SPIβ cells is associated with a heat-sensitive antagonist secreted into the medium, which is accompanied with the impairment of survival pathways.

Introduction

The mammalian phosphatidylinositol transfer protein β (PI-TPβ) is a highly conserved protein with up to 99% sequence identity between species [1, 2]. It shares 77% sequence identity with its isoform PI-TPα. PI-TPβ is mainly associated with the Golgi system and may be the functional analogue of Sec14p, the major yeast PI-TP [3, 4]. Although its physiological function has not yet been established, its importance follows from the observation that gene ablation of PI-TPβ in murine embryonic stem cells prevents embryonic development [5]. Murine embryos lacking PI-TPα develop normally but die within two weeks after birth [6]. This clearly shows that these two isoforms serve different functions in the cell.

In previous studies on wtNIH3T3 cells that have a 10-fold increase in PI-TPβ levels (SPIβ cells) we observed that these cells display a decreased growth rate relative to the wtNIH3T3 cells [7]. In addition, under conditions where sphingomyelin (SM) in the plasma membrane was hydrolyzed to ceramide by exogenous sphingomyelinase, SPIβ cells in contrast to wtNIH3T3 cells maintained the steady-state levels of SM in the plasma membrane suggesting that PI-TPβ was involved in this process [7]. This is in agreement with the finding that *in vitro* PI-TPβ binds and transfers SM in addition to PI and PC while PI-TPα lacks the ability to transfer SM [8]. Unlike SPIβ cells, mutant SPIβS262A cells were unable to instantaneously replenish SM after its degradation by exogenous sphingomyelinase, strongly suggesting that the phosphorylation of Ser-262 was required [9, 10]. If SM replenishment is linked to membrane vesicle flow it could be that PI-TPβ plays a role in the budding process, an activity analogous to Sec14p function in yeast [11].

In contrast to SPIβ cells, cells with an increased expression of PI-TPα (SPIα cells) demonstrate a highly increased rate of proliferation as well as an increased survival upon

induction of apoptosis [12, 13]. Furthermore, it was shown that SPIα cells produce a PI-TPα/COX-2-dependent mitogenic and anti-apoptotic factor. Upon secretion, this factor is able to stimulate growth and to promote survival in wtNIH3T3 and SPIβ cells [13]. In agreement with the increased resistance of these cells towards UV-induced apoptosis, the PI-TPα-dependent survival factor induced COX-2 expression in both cell lines. In addition it had been reported that this survival factor is able to prevent apoptosis in rat motor neurons, suggesting a vital role in the central nervous system [14].

In earlier studies we have shown that SPIβ cells, when compared with wtNIH3T3 cells, are very susceptible towards UV-induced apoptosis, whereas the SPIβS262A cells behave similar to wtNIH3T3 cells [15]. Here we report that two abundant COX-1,-2 dependent arachidonic acid metabolites, PGE_2 and $PGF_{2\alpha}$ do not protect SPIβ cells against apoptosis whereas wtNIH3T3 and SPIβS262A cells are protected. In addition, we report that conditioned medium (CM) from SPIβ cells contains a heat-labile antagonist masking the survival factor present. This antagonist prevents the activation of the anti-apoptotic p42/p44 MAP kinase pathway and the upregulation of COX-2.

Materials and Methods

Materials

Prostaglandins PGE_2 and $PGF_{2\alpha}$ were obtained from Sigma, polyclonal antibodies against COX-1/COX-2 from Cayman; anti-p42/p44 MAP kinase antibodies where obtained from Cell Signaling technology, DMEM and NCS from Invitrogen.

Cell culture

All cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% newborn calf serum (NCS) and buffered with 44 mM $NaHCO_3$. Cells were maintained at 7.5% CO_2 at 37°C in a humidified atmosphere.

Preparation of conditioned medium

Cells were grown to 90% confluency in 150 cm^2 dishes. After washing the cells twice with PBS, the medium was replaced with 13 ml of DBB medium. After 24 h the medium was collected and centrifuged (10 min at 1000 rpm) to remove floating cells. The supernatant is the conditioned medium (CM). Neutral lipids were extracted from CM with two volumes of ethyl acetate (after adjusting to pH 2.0 with formic acid) [16]. CM was heat-denatured by incubation for 20 min at 80°C, then centrifuged for 10 min at 17500xg and the supernatant used for experiments. Under standard conditions 90% confluent cells were incubated with CM derived from an identical surface of cells (i.e. 9.5 cm^2 of cells per well of a six-well dish was incubated with the amount of CM or neutral lipid extract derived from 9.5 cm^2 of cells).

Induction of apoptosis by UV irradiation

Cells were seeded in 6-well plates and grown for 48 h until ca 85% confluency. Before UV treatment, the cells were incubated in DMEM containing 0.1% bovine serum albumin (DBB medium). To investigate the effects on the sensitivity to apoptosis, cells were incubated with CM or prostaglandins in DBB for 4 h prior to UV irradiation. The medium was removed and the cells were given a UV dose (200 J/m^2) using a Stratalinker (Stratagene). After UV irradiation, the cells were incubated with DBB at 37°C. At the indicated time points cell death was morphologically determined as the percentage of cells that are in the process of blebbing.

Determination of COX-1 and COX-2 levels

Cells were grown in 21 cm^2 dishes to 80-90% confluency. Cells were washed twice with PBS and lysed in 20 mM Tris-HCl pH 7.5 containing 0.1% (v/v) NP_{40}. The cell lysate was centrifuged at 17,500xg for 10 min at 4°C and the protein content of the supernatant fraction determined using the Bradford assay [17]. Equal amounts of supernatant proteins (50 μg) were subjected to SDS-PAGE on a 12% gel and Western

blot analysis was performed using an antibody specific for COX-1 or COX-2. The levels of COX-1 or –2 on the immunoblot were quantified using a Bio-Rad GS700 imaging densitometer equipped with an integrating program. In some experiments prior to harvesting the cells were incubated with CM for 5 h. To ensure that identical amounts of protein were analyzed, blots were checked by ponceau S staining.

Determination of p42/44 MAP kinase levels

Cells were grown in 21 cm^2 dishes to 80-90% confluency. Cells were washed twice with PBS and lysed in 20 mM Tris-HCl pH 7.5 containing 0.1% (v/v) NP_{40}, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1 mM aprotinin and 1 mM PMSF. Sample preparation was performed as described above. Western blot analysis was performed using an antibody specific for p42/44 MAP kinase and immunoreactive bands were quantified. In some experiments prior to harvesting the cells were incubated with CM for 10 min. Loading control was performed by ponceau S staining of the blot.

Measurement of ceramide and sphingomyelin levels

Cells were grown to 80% confluency and total lipids were extracted by the method of Bligh and Dyer [18]. Ceramide levels were determined using the *Escherichia coli* diacylglycerol kinase assay as described [19]. Briefly, the lipids were incubated at room temperature for 30 min in the presence of β-octylglucoside/dioleoyl-phosphatidyl glycerol micelles, 2 mM dithiothreitol, 5 μg of proteins from the diacylglycerol kinase membranes, and 2 mM ATP (mixed with [γ-^{32}P]ATP) in a final volume of 100 μl. After Bligh and Dyer extraction the lipids were separated by thin layer chromatography (TLC) in chloroform:acetone:methanol:acetic acid:H_2O (50:20:15:10:5, by vol.) and the radioactivity associated with ceramide-phosphate was measured. Ceramide levels were quantified using external standards and were normalized to phosphate. SM levels were determined as described previously [7].

Results

Apoptosis *sensitivity of wtNIH3T3, SPIβ and SPIβS262A cells*

Previously we have shown that SPIβ cells are sensitive towards UV-induced apoptosis [15]. Exposure of the wtNIH3T3, SPIβ and SPIβS262A cells to other apoptotic stimuli (i.e. 10 ng/ml TNFα, 2.5 μg/ml; serum starvation) showed that the increased apoptotic response of the SPIβ cells was not restricted to UV irradiation. Under all conditions tested, SPIβ cells showed a significantly higher extent of apoptosis than wtNIH3T3 and SPIβS262A cells (Figure 1A).

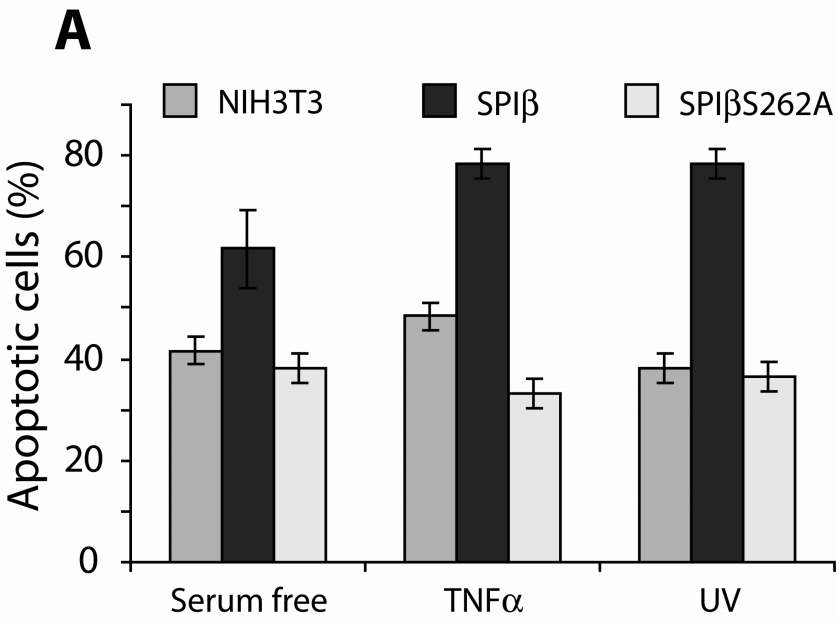

Figure 1 A. Survival of wtNIH3T3, SPIβ and SPIβS262A cells upon induction of apoptosis by UV irradiation, TNFα or serum deprivation. Cells were grown to 90% confluency. Serum starvation: growth medium was replaced by DMEM/Bic/0.1% bovine serum albumin (DBB) and the cells were incubated for 16 h at 37°C. TNFα induced apoptosis: growth medium was replaced by DBB containing cycloheximide (2.5 μg/ml) and TNFα (10 ng/ml) and the cells were incubated for 7 h at 37°C. UV-induced apoptosis: growth medium was replaced by DBB and cells were incubated for 4 h at 37°C. After removal of DBB the cells were irradiated with 200 J/m^2, fresh DBB was added to the cells and incubated for 3 h at 37°C. The number of apoptotic cells (blebbing) was determined by visual analysis. Results ± SD represent the mean values of at least three experiments.

Because SM metabolism may play a role in apoptosis [20-23] , we analyzed SM and ceramide in SPIβ, SPIβS262A and wtNIH3T3 cells. In agreement with previous studies [7], SM levels were comparable in the three cell lines, (i.e. 59 pmol/nmol of total lipid). Similar to other studies, we observed that SM separated in two bands by TLC [24, 25]. The lower band represents short-chain (C16:0) and the upper band long-chain (C24:0/1) SM species. However, we noticed that the relative proportion of the two classes of SM species was different in the three cell lines (Figure 1B). Specifically, wtNIH3T3 and SPIβS262A cells have relatively more short chain than long chain SM species (ratio of long chain over short chain of 0.7), whereas SPIβ cells have relatively less short chain than long chain SM species (ratio of 1.25). Ceramide analysis of wtNIH3T3 and SPIβ cells showed that the species composition was similar to that of SM (data not shown).

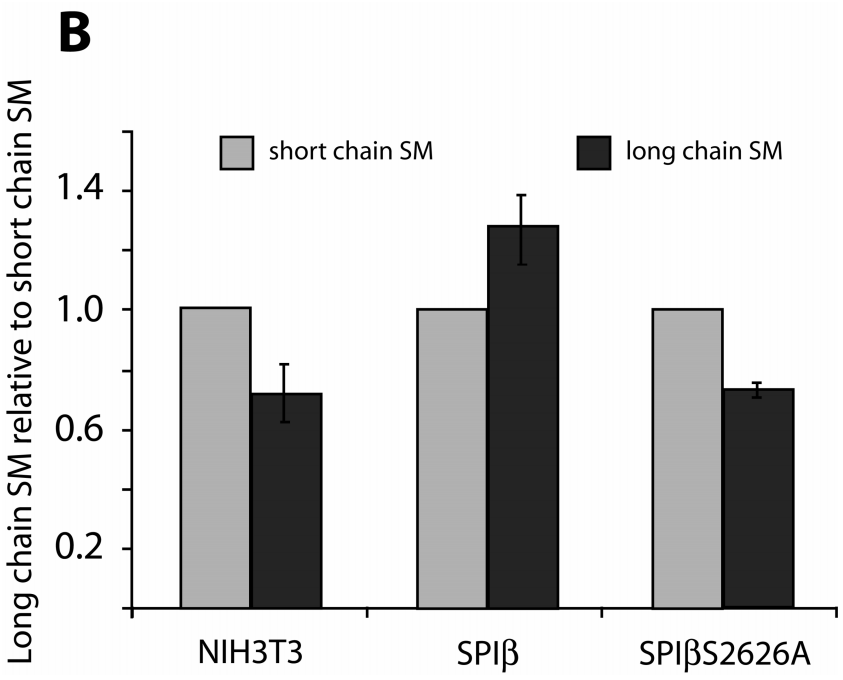

Figure 1B. Long and short chain SM levels in wtNIH3T3, SPIβ and SPIβS262A cells. Cells were grown to 80% confluency and total lipids were extracted by the method of Bligh and Dyer [18]. Results ± SD represent the mean values of at least three experiments

In agreement with previous observations PGE_2 and $PGF_{2\alpha}$ are able to protect wtNIH3T3 mouse fibroblast cells against apoptosis (Figure 2A) [26, 27]. Incubation of SPIβ cells with these prostaglandins did not protect these cells against UV-induced

apoptosis, whereas CM from wtNIH3T3 cells did protect (Figure 2B). The concentration of PGE_2 and $PGF_{2\alpha}$ used were 0.5 and 0.1 ng/ml. These concentrations are comparable to the levels detected in CM from SPIα cells and are 5-fold higher compared to the levels detected in CM from NIH3T3 and SPIβ cells [13]. A similar protection was observed when neutral lipid extracts from this CM were used (data not shown). Similar to wtNIH3T3, SPIβS262A cells were protected by both PGE_2/$PGF_{2\alpha}$ and CM from wtNIH3T3 cells (Figure 2A-C).

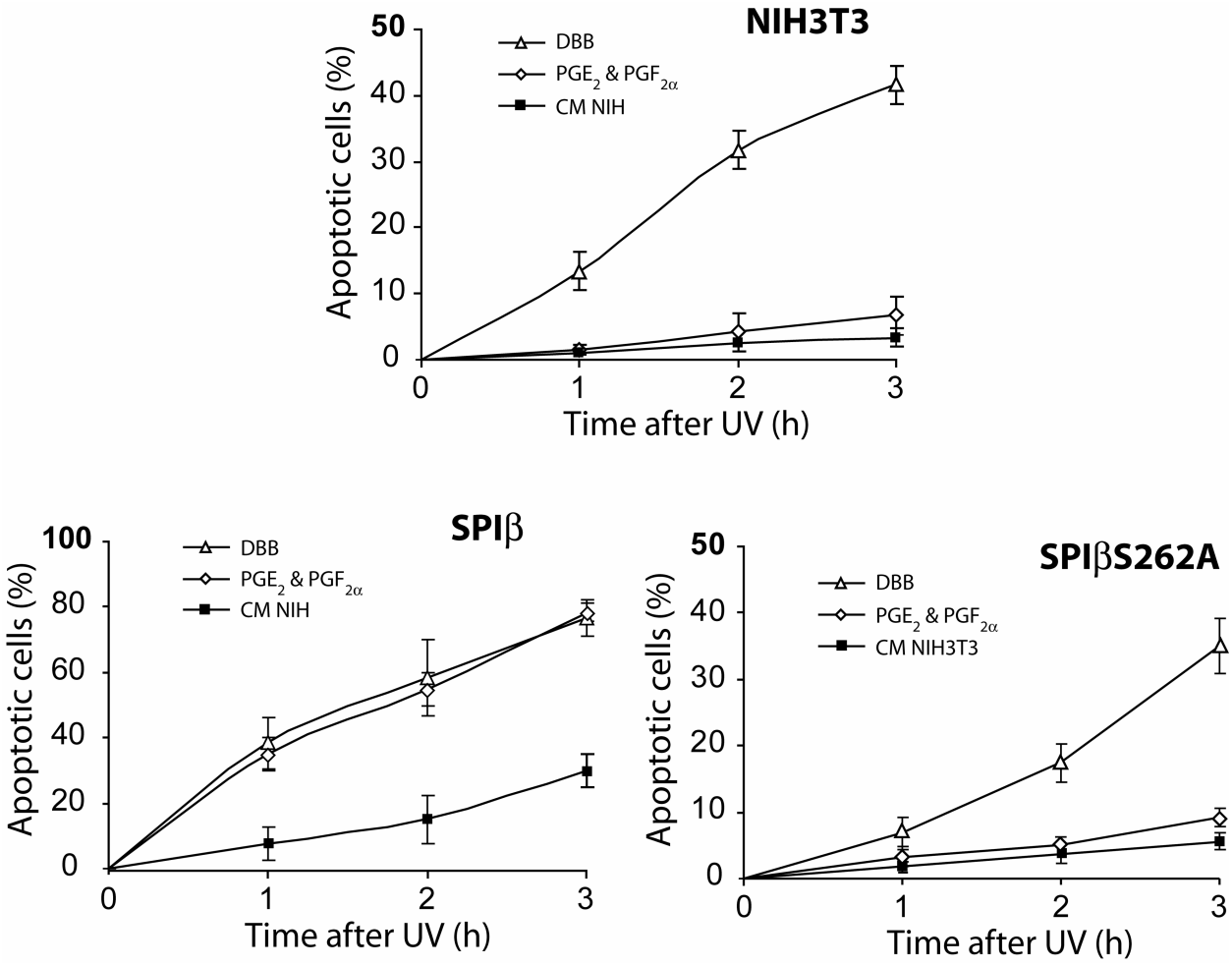

Figure 2. Survival of wtNIH3T3, SPIβ and SPIβS262A cells upon induction of apoptosis by UV irradiation. Cells were grown to 90% confluency. The growth medium was replaced by DMEM/Bic/0.1% bovine serum albumin (DBB), DBB containing PGE_2 (0.5 ng/ml) and $PGF_{2\alpha}$ (0.1 ng/ml) or CM from wtNIH3T3 cells and the cells were incubated for 4 h at 37 °C. After removal of DBB or CM, the cells were irradiated with 200 J/m^2. Fresh DBB was added to the cells and the number of apoptotic cells (blebbing) was determined by visual analysis at the indicated times. Results ± SD represent the mean values of at least three experiments performed in duplicate.

Expression of cyclooxygenase-1 and -2

The anti-apoptotic activity of CM from wtNIH3T3 and SPIα cells is an eicosanoid, the synthesis of which is (partially) dependent on COX-2 activity [13]. As shown by Western blot analysis, the level of COX-2 in SPIβ cells is reduced when compared to wtNIH3T3 cells (Figure 3A; lane 1 and 4). Again the SPIβS262A cells resembled the wild-type cells as the levels of COX-2 were comparable in both cell lines (Figure 3A; lane 1 and 7). Arachidonic acid metabolites produced by COX-2 can stimulate the expression of this enzyme via an autocrine pathway [28, 29]. By using an ELISA kit we showed that the amount of PGE_2 secreted by the SPIβ cells is equal to that of wtNIH3T3 cells. Therefore the reduced level of COX-2 in SPIβ cells is not linked to a decreased level of PGE_2. When SPIβ cells were incubated for 5 h with PGE_2 in combination with $PGF_{2\alpha}$, COX-2 levels remained the same (Figure 3A; lane 4 and 5), whereas the COX-2 levels of wtNIH3T3 and SPIβS262A cells were increased (Figure 3A; lanes 2 and 8). This strongly suggests that the upregulation of COX-2 by PGE_2/$PGF_{2\alpha}$ is inhibited in SPIβ cells. On the other hand, incubation of SPIβ cells with CM from wtNIH3T3 cells did increase the COX-2 levels (Figure 3A; cf. lanes 4 and 6). A similar upregulation of COX-2 was observed for wtNIH3T3 cells (Figure 3A; cf. lanes 1 and 3) and SPIβS262A cells (Figure 3A; cf. lanes 7 and 9). This suggests that the upregulation of COX-2 in SPIβ cells by CM from wtNIH3T3 cells may be linked to the increased survival upon UV irradiation under the same conditions. Lack of COX-2 induction by PGE_2 and $PGF_{2\alpha}$ in SPIβ cells agrees with the failure to protect these cells (Figure 2B). In contrast to COX-2, expression levels of COX-1 were similar in all three cell lines and did not change upon incubation with PG's or CM from wtNIH3T3 cells (data not shown)

p42/p44-MAP kinase activation

Activation of p42/p44 MAP kinase is commonly observed after hormone or polypeptide growth factor induced proliferation or cell survival [30-33]. Incubation of wtNIH3T3, SPIβ and SPIβS262A cells with CM from wtNIH3T3 cells induced a rapid

activation of p42/p44 MAP kinase (Figure 3B; cf. lanes 3, 6, 9). Upon incubation with PGE_2 and $PGF_{2\alpha}$, p42/p44 MAP kinase was activated in wtNIH3T3 and SPIβS262A cells but not in SPIβ cells (Figure 3B; cf. lanes 2, 5, 8). These data support the finding that PGE_2 and $PGF_{2\alpha}$ do protect wtNIH3T3 and SPIβS262A cells against apoptosis but fail to protect SPIβ cells, indicating that in SPIβ cells this signal pathway for stimulation of proliferation and cell survival is inhibited.

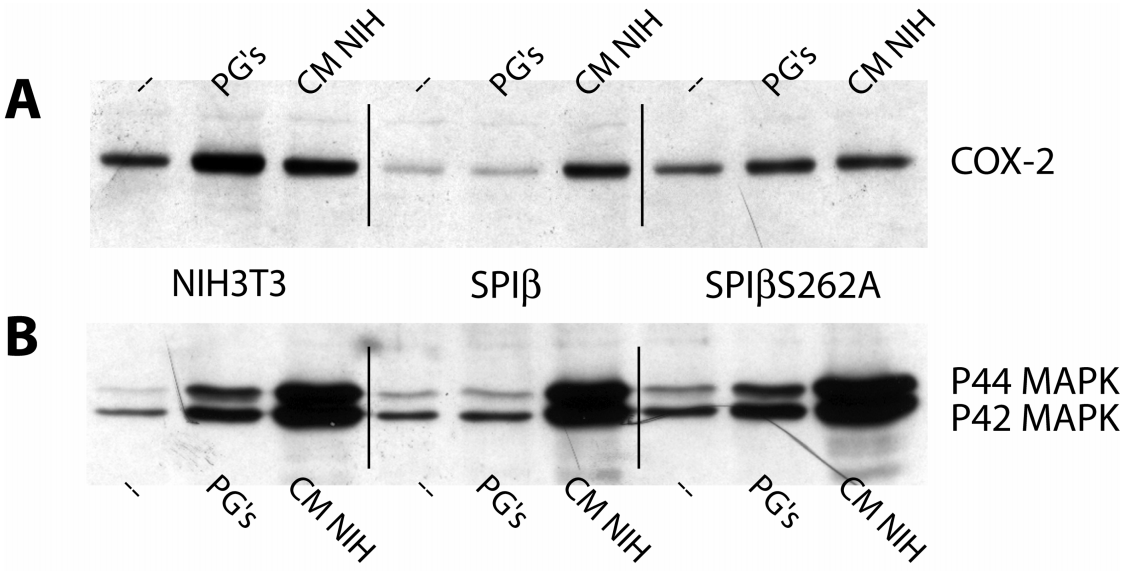

Figure 3A. Expression of COX-2 in wtNIH3T3, SPIβ and SPIβS262A upon incubation with $PGE_2/PGF2_\alpha$ or CM from wtNIH3T3 cells. Cells were grown to 90% confluency and incubated with DMEM/Bic/0.1% bovine serum albumin (DBB), DBB containing PGE_2 (0.5 ng/ml) and $PGF_{2\alpha}$ (0.1 ng/ml) or CM from wtNIH3T3 cells for 5 h at 37°C. Equal amounts of cell lysate protein (30 µg) from NIH3T3, SPIβ and SPIβS262A cells were subjected to SDS PAGE followed by Western blot analysis using a COX-2 specific antibody. Representative experiment performed in triplicate. Loading control was performed by ponceau S staining of the blot.

Figure 3B. Phosphorylation of p42/p44 MAP kinase in wtNIH3T3, SPIβ and SPIβS262A upon incubation with $PGE_2/PGF_{2\alpha}$ or CM from wtNIH3T3 cells. Cells were grown to 90% confluency and incubated with DMEM/Bic/0.1% bovine serum albumin (DBB), DBB containing PGE_2 (0.5 ng/ml) and $PGF_{2\alpha}$ (0.1 ng/ml) or CM from NIH3T3 cells for 10 min at 37°C. Equal amounts of cell lysate protein (25 µg) from NIH3T3, SPIβ and SPIβS262A cells were subjected to SDS-PAGE followed by Western blot analysis using a p42/p44 MAP kinase specific antibody. Representative experiment performed in triplicate. Loading control was performed by ponceau S staining of the blot.

Secretion of an antagonist of anti-apoptotic activity

SPIα cells secrete a highly potent anti-apoptotic and mitogenic factor(s) [12, 13]. Although to a lesser extent, wtNIH3T3 cells also produce bioactive factors. Indeed, when prior to UV irradiation SPIβ cells were incubated with CM from wtNIH3T3 cells instead of DBB, the extent of apoptosis was decreased by 45% (Figure 4). Incubation of SPIβ cells with CM from SPIβ cells did not prevent apoptosis. However, upon heat treatment (20 min at 80°C) CM from SPIβ cells expressed an anti-apoptotic activity comparable to that from wtNIH3T3 cells (Figure 4). As a control, heat treatment of CM from wtNIH3T3 and SPIβS262A cells had little effect on the anti-apoptotic activity indicating that this activity was heat stable. This strongly suggests that CM from SPIβ cells contains a heat-labile factor (antagonist) that interferes with the anti-apoptotic activity present.

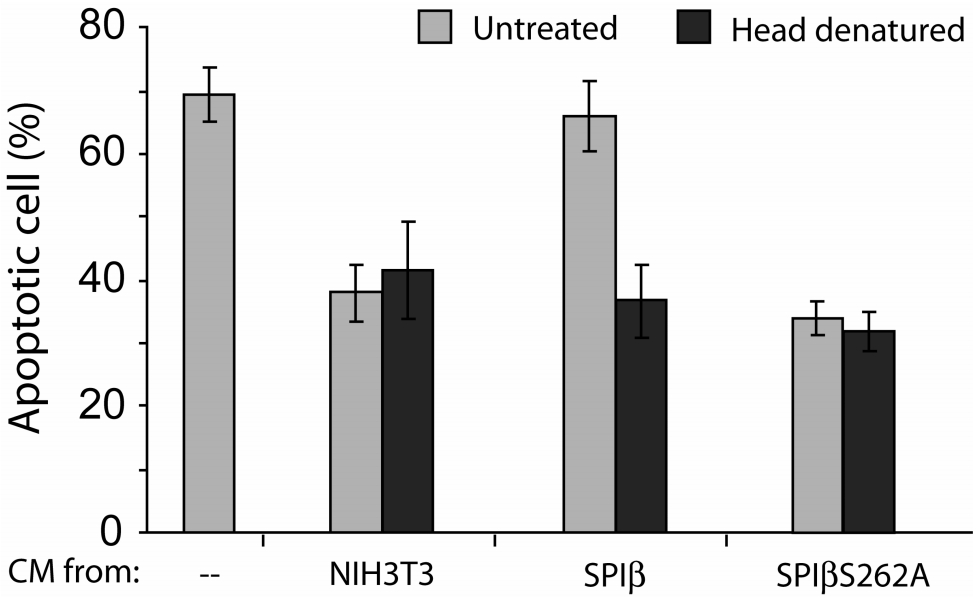

Figure 4. Survival of SPIβ cells upon induction of apoptosis by UV irradiation after preincubation with CM from wtNIH3T3, SPIβ and SPIβS262A cells with or without heat denaturation. SPIβ cells were grown to 90% confluency. The growth medium was replaced by DMEM/Bic/0.1% bovine serum albumin (DBB) or by CM and the cells were incubated for 4 h at 37 °C. After removal of DBB the cells were irradiated with 200 J/m^2, fresh DBB was added to the cells and incubated for 3 h at 37^{o}C. The number of apoptotic cells (blebbing) was determined by visual analysis. Results ± SD represent the mean values of at least three experiments performed in duplicate.

The anti-apoptotic activity of CM from SPIβS262A cells resembled that of wtNIH3T3 cells indicating that phosphorylation of PI-TPβ is required for the production of the antagonist.

In agreement with the observations for cell survival, CM from SPIβ cells after heat-treatment (20 min 80°C) induced COX-2 in SPIβ cells (Figure 5A; cf. lanes 2 and 6) to the same extent as CM from wtNIH3T3 cells (Figure 5A; cf. lanes 4 and 6) and SPIβS262A cells (Figure 5A; lanes 6 and 8) whereas the untreated CM from SPIβ cells had no effect on COX-2 expression (Figure 5A; lane 5). Similarly, the untreated CM

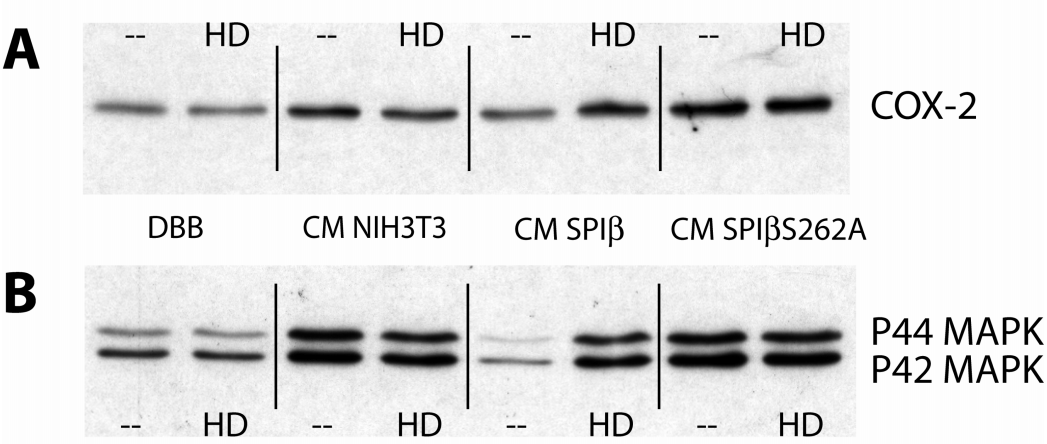

Figure 5A. Expression of COX-2 in SPIβ cells upon incubation with CM from wtNIH3T3, SPIβ and SPIβS262A with or without heat denaturation of the CM. Cells were grown to 90% confluency and incubated for 5 h at 37°C with CM from NIH3T3, SPIβ or SPIβS262A cells, either heat denatured or not. Equal amounts of cell lysate protein (30 µg) from NIH3T3, SPIβ and SPIβS262A cells were subjected to SDS PAGE followed by Western blot analysis using a COX-2 specific antibody. Representative experiment performed in triplicate. Loading control was performed by ponceau S staining of the blot.

Figure 5B. Phosphorylation of p42/p44 MAP kinase in SPIβ cells upon incubation with CM from wtNIH3T3, SPIβ and SPIβS262A with or without heat denaturation of the CM. Cells were grown to 90% confluency and incubated for 10 min at 37°C with CM from wtNIH3T3, SPIβ or SPIβS262A cells, either heat denatured or not. Equal amounts of cell lysate protein (25 µg) from NIH3T3, SPIβ and SPIβS262A cells were subjected to SDS PAGE followed by Western blot analysis using a p42/p44 MAP kinase specific antibody. Representative experiment performed in triplicate. Loading control was performed by ponceau S staining of the blot.

from SPIβ has no effect on the level of p42/p44 MAP kinase, whereas the heat-treated CM from SPIβ cells is able to activate MAPK (Figure 5B; cf. lanes 5 and 6). For comparison, the untreated CM from wtNIH3T3 and SPIβS262A cells are able to activate MAPK in SPIβ cells (Figure 5B; cf. lanes 3, 4, 7 and 8), resulting in protection of these cells against UV induced apoptosis (Figure 4).

The data are summarized in figure 6.

Discussion

Previously we have shown that a ten-fold increase of PI-TPβ in wtNIH3T3 mouse fibroblast cells (SPIβ cells) significantly increases the sensitivity towards apoptosis induced by UV irradiation [15]. Here we show that SPIβ cells incubated with TNFα and serum starvation are much more prone to apoptosis indicating that the increased apoptotic response was not restricted to UV irradiation. A role of PI-TPβ in cell survival was also indicated by the finding that initially, using another expression vector (pSG5) than the one currently used (pBK-CMV) we failed to obtain stable SPIβ cell lines. Due to high levels of PI-TPβ we routinely observed that these cells died after 4-5 passages. Previously we have shown that PI-TPβ is mainly associated with the Golgi system and that this association requires the PKC-dependent phosphorylation of Ser-262 as the mutant PI-TPβ(S262A) is present throughout the cell [3, 9, 10]. By overexpressing PI-TPβ(S262A) to a level comparable to that of PI-TPβ (9.0 and 10.6 ng per 100 μg cytosolic protein, respectively) the ensuing SPIβS262A cells have a sensitivity towards apoptosis comparable to that of wtNIH3T3 cells [15]. This strongly suggests that there is a relationship between the phosphorylation of PI-TPβ and the sensitivity towards apoptosis.

Earlier studies from our laboratory have shown that PI-TPβ is phosphorylated *in vitro* as well as *in vivo* on Ser-166 and Ser-262. Michaelis-Menten analysis showed that Ser-262 is the major phosphorylation site [9, 15]. In agreement with this, *in situ* PI-TPβ is constitutively phosphorylated on Ser-262 [9]. Given that in contrast to PI-TPβS262A, the

overexpressed PI-TPβ is associated with the Golgi system, we infer that the apoptosis sensitivity is linked to the Golgi localization. Recent studies showed that the Golgi localization of PI-TPβ is not dependent on the phosphorylation of Ser-262 [34, 35]. Although we have no explanation for this discrepancy we do find that increased levels of PI-TPβS262A have no effect on the apoptosis sensitivity. Thus in case that PI-TPβS262A is still associated with the Golgi, phosphorylation of Ser-262 is required for this protein to affect apoptosis.

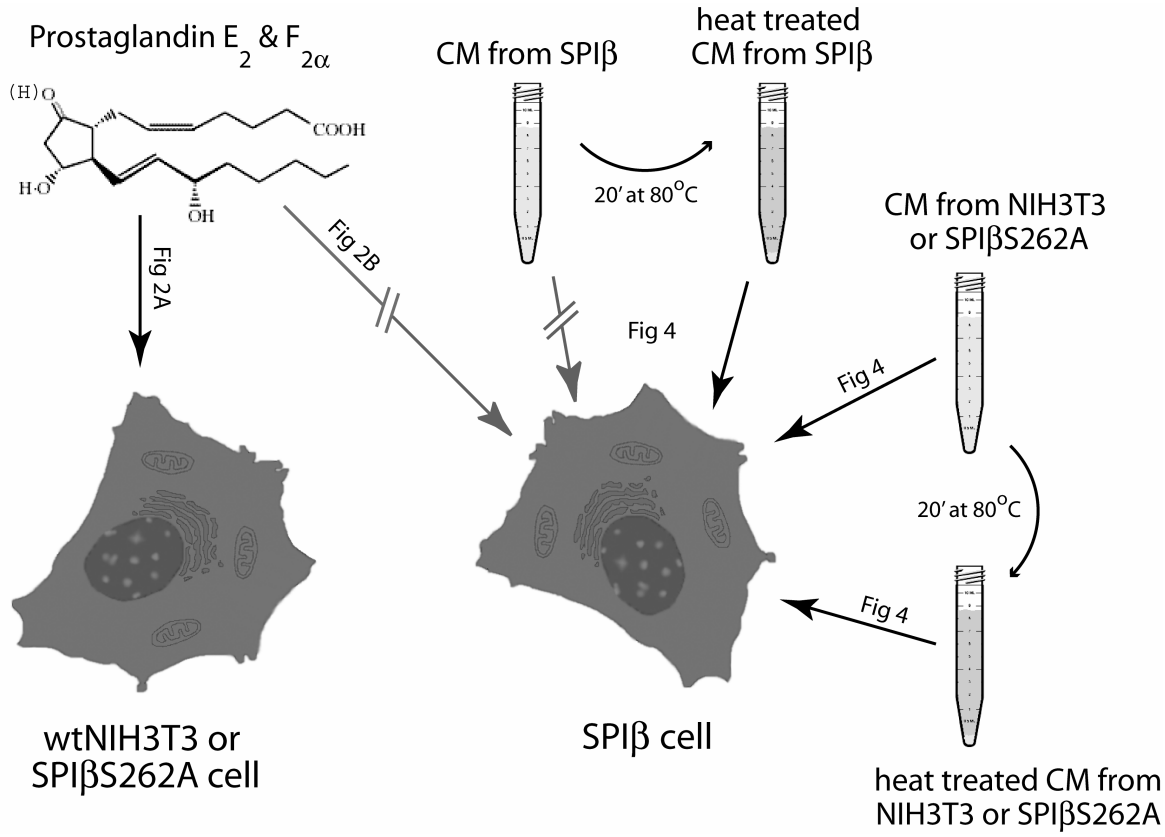

Figure 6. Summarizing figure. Prostaglandin E_2 and prostaglandin $F_{2\alpha}$ are unable to protect SPIβ cells against UV-induced apoptosis whereas wtNIH3T3 cells are protected. In addition, the anti-apoptotic activity present in CM from SPIβ cells is masked by the presents of an antagonist, which is removed upon heat treatment. The fact that CM from wtNIH3T3 cells and CM from SPIβS262A cells lack this antagonist suggests that the production is dependent on the presents and phosphorylation of PI-TPβ.

Two important parameters linked to apoptosis are activation of p42/p44 MAP kinase and expression of COX-2 [36-38]. In general, inhibition of COX-2 expression enhances apoptosis and more specifically reduces the incidence and progression of tumors in animal models [39-42]. Phosphorylation of p42/p44 MAP kinase through the Ras > Raf > MAP kinase kinase (MKK) cascade is associated with proliferation, protection against apoptosis and angiogenesis [30-33]. Here we show that the prostaglandins PGE_2/$PGF_{2\alpha}$ are unable to upregulate COX-2 in SPIβ cells, but are able to do so in wtNIH3T3 and SPIβS262A cells, showing that the pathway of upregulation is blocked in SPIβ cells (Figure 3A). This results in a lower basal level of COX-2 in SPIβ cells and prevents prostaglandins (PG's) from protecting SPIβ cells against apoptosis. In addition we show that these PG's cannot activate p42/p44 MAP kinase in SPIβ cells whereas this pathway is activated in wtNIH3T3 and SPIβS262A cells (Figure 3B). These observations may explain why SPIβ cells are more prone to apoptosis and why PGE_2 and $PGF_{2\alpha}$ are unable to induce survival in SPIβ cells. As shown before, cells with increased expression of PI-TPα produce and secrete a potent mitogenic and survival factor. As a result the expression of COX-2 in these cells is upregulated [13]. As shown in figure 3A, the COX-2 level in SPIβ cells is reduced when compared with wtNIH3T3 and SPIβS262A cells, whilst the COX-1 level is unaffected. This raises the question how the level of COX-2 is regulated in these cells. The fact that CM from wtNIH3T3 (and from SPIα) cells does promote cell survival via activation of p42/p44 MAP kinase and upregulation of COX-2 shows that the failure of the PGE_2 and $PGF_{2\alpha}$ to do so is not at a transcriptional level but most likely further upstream at the receptor level.

Since PI-TPα levels were similar in SPIβ cells and wtNIH3T3 cells it was unexpected that CM from SPIβ cells appeared to lack the survival activity present in the CM from NIH3T3 cell. Interestingly, CM from SPIβ cells acquired survival activity upon heating, indicating that the expression of PI-TPβ is responsible for the production and secretion of a component that interferes with the action of the intrinsic survival factors present. In this context, the PI-TPα-dependent eicosanoids are of interest [13]. Apparently, these eicosanoids are also produced by SPIβ but are inhibited by the PI-TPβ-dependent 'antagonist' either by direct interaction or by interfering with the activation of the GPCR

[13]. Attempts to gain insight into the nature of the PI-TPβ-dependent 'antagonist' were inconclusive. Experiments using protein synthesis inhibitors and analysis of [^{14}C]serine-labeled SM metabolites secreted by SPIβ cells revealed no significant differences compared with control cells.

The relationship between SM metabolism and apoptosis has been investigated extensively. B-cell receptor-triggered apoptosis is associated with an early rise of C16 ceramide leading to the subsequent formation of long chain C24 ceramide via activation of effector caspases [43]. Furthermore, it was shown that the NO donor sodium nitroprusside (SNP) dose-dependently decreased MC3T3-E1 osteoblast viability through enhancing the release of intracellular C22 and C24 ceramide leading to apoptosis [44]. Apoptotic stimuli including TNFα [45], ionizing radiation [46] and B-cell receptor cross-linking [43] can generate ceramide by the induction of SM hydrolysis through the action of sphingomyelinases or the *de novo* pathway. Specifically, ceramide formed in mitochondria by selective hydrolysis of a mitochondrial pool of SM induces cell death [47]. In contrast to SPIβS262A and wtNIH3T3 cells, SPIβ cells maintain the total level of SM under conditions where SM is degraded in the plasma membrane to ceramide by exogenous sphingomyelinase [7, 9]. Although the mechanism by which PI-TPβ regulates the rapid conversion of ceramide to SM is not known, the data again strongly suggest that PI-TPβ must be phosphorylated at Ser-262 in order to maintain the cellular SM levels. Although increased levels of PI-TPβ appear to be required for SPIβ cells to rapidly convert ceramide to SM, we found that steady-state levels of ceramide in SPIβ and wtNIH3T3 cells are similar. However, we did observe that the molecular species of the fatty acids of SM (Figure 1B) and ceramide are different with SPIβ cells having relatively less short chain (C16) and more long chain species (C24:1/0) compared with wtNIH3T3 cells. To date it is not known whether the sensitivity towards apoptosis of SPIβ cells is related to this shift from short chain to long chain ceramide/SM. However, it could be that the relative enrichment of long chain SM species in SPIβ cells has an effect on plasma membrane properties [48]. To what extent this may affect ceramide production in response to apoptosis stimuli remains to be investigated.

We propose that as a consequence of an antagonist blocking of the autocrine action of the PI-TPα-dependent survival factor, the SPIβ cells are more sensitive towards induced apoptosis (summarized in fig 6). In addition, the failure of PGE_2/$PGF_{2\alpha}$ to both activate p42/p44 MAP kinase and to upregulate COX-2 levels may also explain why SPIβ cells are more prone to apoptosis (summarized in fig 6). At this point our data suggest that since an increased expression of PI-TPβ promotes apoptosis, the deletion of PI-TPβ may have a prohibitive effect on apoptosis. Since apoptosis is an essential event during early embryonic development, the proposed role of PI-TPβ in apoptosis may explain why the generation of a PI-TPβ knock-out mouse has failed [6]. Understanding why and how expression of a single protein decreases the rate of proliferation as well as survival of cells might be of interest for research on methods to decrease the growth of rapidly proliferating tumor cells that have gained resistance against induction of apoptosis.

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Chapter 5

Characteristics of the anti-apoptotic factors present in CM from mouse fibroblast cells overexpressing PI-TPα

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Introduction

Mouse fibroblast cells with an increased expression of phosphatidylinositol transfer protein α (PI-TPα) show an enhanced phospholipase A (PLA)-mediated degradation of phosphatidylinositol (PI). In these so-called SPIα cells increased levels of lysophosphatidylinositol (lysoPI), glycerophosphoinositol, inositol 1-phosphate (I(1)P), and inositol 2-phosphate (I(2)P) are found relative to NIH3T3 control cells. Since PI is highly enriched in arachidonic acid, a significant amount of arachidonic acid is released [1]. Arachidonic acid or its metabolites are the main precursors in the synthesis of biologically active eicosanoids including prostaglandins, prostacyclins, prostaglandin ethanolamines and prostaglandin glycerol esters. In line with this, SPIα cells produce and secrete an eicosanoid-like mitogenic and anti-apoptotic factor(s) [2] (Chapter 2). This anti-apoptotic activity present in a cell-free conditioned medium (CM) from SPIα cells is able to induce survival in wild-type NIH3T3 (wtNIH3T3) mouse fibroblast cells, SPIβ cells (i.e. wtNIH3T3 cells overexpressing PI-TPβ) by activation of the p42/p44 MAP kinase and Akt/PKB pathway [2] (chapter 3). In addition, primary spinal cord-derived motor neurons, human neutrophils and human embryonic kidney cells were protected against apoptosis [3, 4](unpublished data). Previous studies on the identity of the PI-TPα-dependent anti-apoptotic factors showed that the production is partially dependent on cyclooxygenase 2 (COX-2). Thin layer chromatography scans of the neutral lipid extracts of CM from wtNIH3T3 and SPIα cells, labeled to equilibrium with [^{14}C]arachidonic acid, revealed at least three additional COX-2-dependent arachidonic acid metabolites present in CM from SPIα cell. Furthermore, the lipid extract of CM from SPIα cell was almost completely devoid of free arachidonic acid indicating a very active metabolism of this fatty acid [2].

In this paper we further characterized the anti-apoptotic factors produced and secreted by SPIα cells using several purification steps, competition studies and extensive mass spectrometry (MS) analysis.

Materials and methods

Materials

All solvents were acquired from Sigma-Aldrich. Deuterated standards ([$^2H_4$]-2-arachidonoylglycerol (2AG), [$^2H_4$]-oleoylethanolamide (OEA), [$^2H_4$]-palmitoylethanolamide (PEA) and [$^2H_4$]-arachidonoylethanolamide) were obtained from Cayman Chemical (Ann Arbor, MI) and Centricon centrifugal filter unit from Millipore.

Cell culture

All cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% newborn calf serum (NCS) and buffered with 44 mM $NaHCO_3$. Cells were maintained at 7.5% CO_2 at 37°C in a humidified atmosphere.

Preparation of conditioned medium

Cells were grown to 90% confluency in 150 cm^2 dishes. After washing the cells twice with PBS, the medium was replaced with 13 ml of DMEM containing 0.1% bovine serum albumin (DBB medium). After 24 h the medium was collected and centrifuged (10 min at 1000 rpm) to remove floating cells. The supernatant is the conditioned medium (CM). Lipids were extracted from CM either by two volumes of ethyl acetate (after adjusting to pH 2.0 with formic acid) (neutral lipid extraction) [5] or by methanol/chloroform (1:2, v/v) extraction [6].

To determine anti-apoptotic activity, 90% confluent SPIβ cells or wtNIH3T3 cells were incubated with CM derived from an identical surface of cells (i.e. 9.5 cm^2 of cells per well of a six-well dish was incubated with the amount of CM or neutral lipid extract derived from 9.5 cm^2 of cells).

Induction of apoptosis by UV irradiation

Cells were seeded in 6 well plates and grown for 48 h until ca 90% confluency. Before UV irradiation, the cells were incubated in DMEM containing 0.1% bovine serum albumin (DBB medium). To investigate the effects on the sensitivity to apoptosis, cells were incubated with CM or column fractions for 4 h prior to UV irradiation. The medium was removed and cells were irradiated (200 J/m^2) using a Stratalinker (Stratagene). After UV irradiation, the cells were incubated with DBB at 37°C for 3 h. Cell death was morphologically determined as the percentage of cells that are in the process of blebbing.

Proteins separation

Proteins in CM from SPIα cells were separated by a Centricon centrifugal filter unit (low binding Ultracel-YM-30) with a 30 kDa cut-off spun at 5000xg at 4^oC for 1 h. Before further use, the fractions in both the collection and filter tube were restored to the original volume.
To separate proteins by gel filtration, the volume of CM was reduced from 13 ml to 2.5 ml by a Centricon centrifugal filter unit (low binding Ultracel-YM-3) with a 3 kDa cut-off without losing activity. The samples were loaded on a SS-100 column (50 x 2 cm) and fractions of 3 ml were collected. To determine anti-apoptotic activity, 1.5 ml of these fractions was used.

Reversed phase chromatography

We used an SMART (HPLC) system coupled to a UV detector (Pharmacia Biotech). The ethyl acetate extracts of CM were separated using a C18 reversed phase column (Alltech, 30 mm x 4,6 mm), eluted with a gradient of acetonitrile (95%) in water (4.2 min 0% acetonitrile; from 0% to 95% acetonitrile in 33.3 min; 8.3 min 95% acetonitrile) at a flow rate of 240 µl/min. Acetonitrile (pH 6.0) contained 25 mM of ammonium acetate. Chromatography was carried out at room temperature. UV detection was at 214, 254 and

280 nm. The radioactive label present in the fractions eluted from the reversed phase column was measured by scintillation counting.

Fatty acid metabolite extraction

Arachidonoylethanolamide (anandamide; AEA) and other fatty acyl ethanolamides were extracted as described by Schreiber, *et al* and Astarita, *et a*l [7, 8]. Briefly, conditioned media were spiked with [$^2H_4$]-2-arachidonoylglycerol (2AG), [$^2H_4$]-oleoylethanolamide (OEA), [$^2H_4$]-palmitoylethanolamide (PEA) and [$^2H_4$]-AEA as internal standard, and subjected to methanol/chloroform (1:2, v/v) extraction. The organic phase was dried under N_2 and reconstituted in chloroform/methanol (1:4, v/v) for further analysis.

To examine fatty acid metabolite composition in wtNIH3T3 and SPIα cells, cells in a 25 cm^2 flask were spiked with as internal standard and scraped of in 2 ml methanol. Lipids were extracted by adding 4 ml chloroform. Phase separation was obtained by addition of 2 ml water. Protein contents was determined in the initial methanol solution using a Bradford protein assay [9]. The chloroform phase was dried under N_2 and the residue dissolved in chloroform/methanol (1:4, v/v) for further analysis.

Fatty acid metabolite LC/MS analyses

Fatty acid metabolites were analyzed by using an 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Palo Alto, CA) equipped with ESI interface. XDB Eclipse C18 column (50 x 4.6 mm ID, 1.8 μm, Zorbax), eluted with a gradient of methanol in water (from 0% to 100% methanol in 2.5 min) at a flow rate of 1.5 ml/min. Methanol contained 0.25% acetic acid and 5 mM of ammonium acetate. Column temperature was kept at 40°C. MS detection was in the positive ionization mode; capillary voltage was set at 3 kV. N_2 was used as drying gas at a flow rate of 13 l/min and a temperature of 350°C. Nebulizer pressure was set at 60 psi. Fatty acids were quantified with an isotope-dilution method [10], monitoring sodium adducts of the molecular ions $[M+Na]^+$ in the selected ion-monitoring (SIM) mode.

HPLC-MS

The system used for HPLC-MS was identical to that used for LC/MS (see above). CM was subjected to methanol/chloroform (1:2, v/v) extraction and separated using a XDB Eclipse C18 column, eluted with a gradient of methanol in water (from 60% to 100% methanol in 15 min; 5 min at 100% methanol; from 100% to 60% methanol in 1 min) at a flow rate of 1 ml/min. Methanol contained 0.25% acetic acid and 5 mM of ammonium acetate. Column temperature was kept at 30°C. MS detection was in the positive ionization mode; full scan from 250 m/z to 500 m/z; capillary voltage was set at 3 kV. N_2 was used as drying gas at a flow rate of 13 l/min and a temperature of 350°C. Nebulizer pressure was set at 60 psi.

Results

Protein separation

Initial protein separation of CM from SPIα cells showed that the bulk of mitogenic and anti-apoptotic activity was retained in the fraction containing proteins heavier than 30 kDa. In addition, protein separation was performed by gel filtration of CM from SPIα cells labeled to equilibrium with [^{14}C]arachidonic acid. Scans of radioactive compounds in fractions collected from the gel filtration column (SS-100) showed [^{14}C] label to co-elute with bovine serum albumin (Figure 1; peak 1). A second peak containing radioactive label was detected (Peak 2), which appeared to contain very little protein. Both peaks were shown to contain anti-apoptotic activity with highest activity detected in the peak co-eluting with bovine serum albumin (data not shown). The neutral lipid extract of both protein peaks was separated by reversed phase chromatography using a C18-RP-column. Scans of [^{14}C] label showed three dominant peaks containing the bulk of radioactive label and a fourth peak (peak IV; figure 2), more hydrophobic than arachidonic acid (peak III), containing a limited amount of label. Peaks III and IV were acquired by subjecting protein peak 1 to ethyl acetate extraction. Subjecting 'protein' peak 2 to extraction revealed peaks I and II. It was shown that standard endocannabinoids

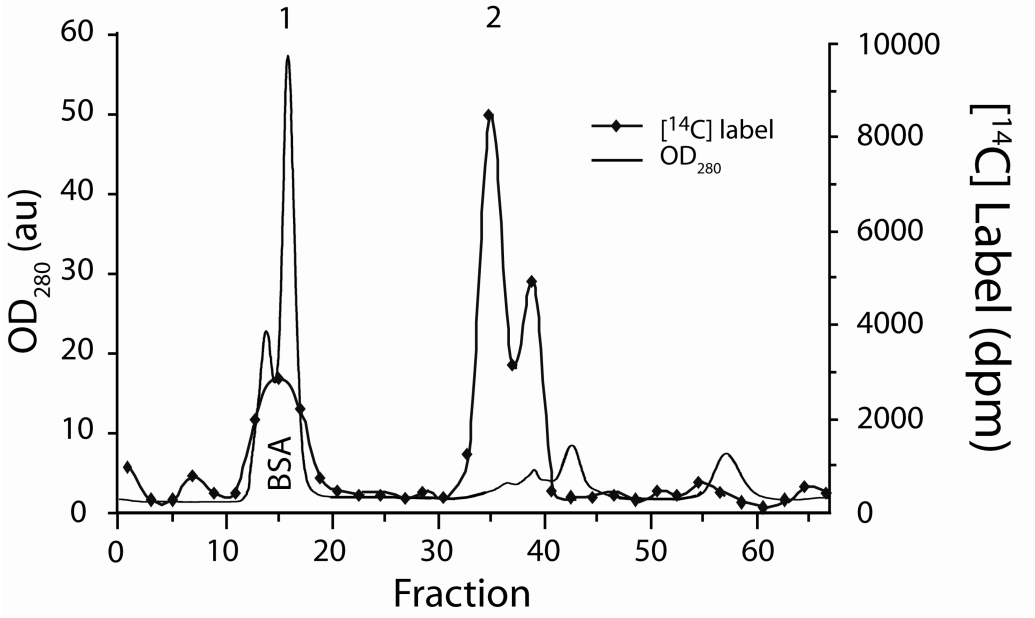

Figure 1. The elution pattern of CM from SPIα cells from a SS-100 gel filtration column. CM from SPIα cells was concentrated by a Centricon centrifugal filter unit. The samples were loaded on a SS-100 column and fractions of 3 ml were collected. The protein content of the fractions was determined by measuring the optical density at 280 nm (OD_{280}). The presence of [^{14}C] label in the fractions was determined by liquid scintillation counting.

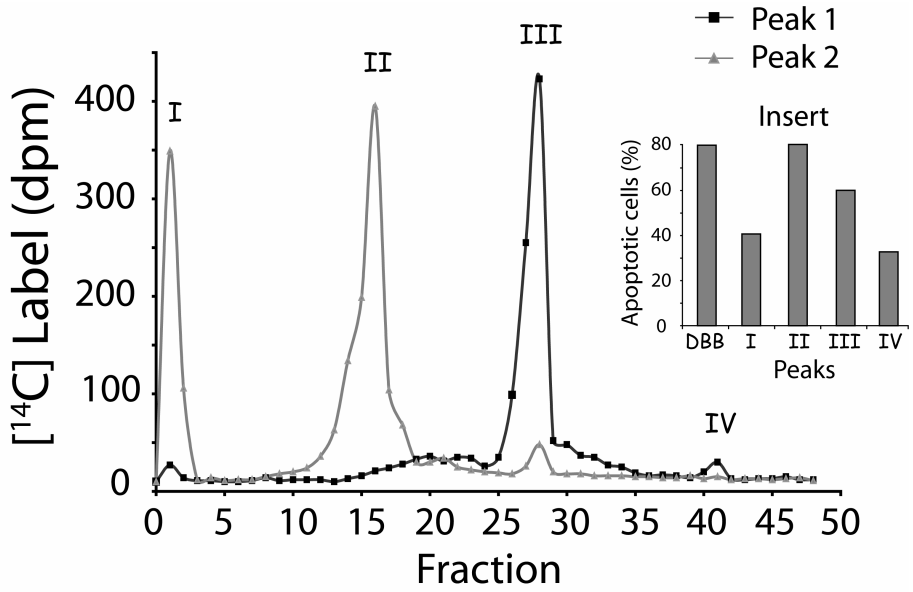

Figure 2. The elution pattern of CM from SPIα cells from a C18 reversed phase column. The peaks containing [^{14}C] label upon separation of CM from SPIα cells on a SS-100 gel filtration column were subjected to ethyl acetate extraction and loaded separately on a C18 reversed phase column. The presence of [^{14}C] label in the fractions was determined by liquid scintillation counting. **Insert: anti-apoptotic activity of fractions eluted from the C18 reversed phase column.** SPIβ cells were incubated for 4 h with DBB or DBB containing fractions eluted from the reversed phase column. Apoptosis was induced by UV irradiation (200 J/m^2) and the number of apoptotic cell was determined by visual analysis after 3 h.

(2-arachidonoylglycerol ether, 2-arachidonoylglycerol ester, and anandamide) eluted close to peak III. Analysis of the four peaks (I,II,III, and IV) for anti-apoptotic activity tested on SPIβ cells revealed that the activity was divided over 3 peaks (figure 2 insert). The fractions in between the four peaks were not analyzed for anti-apoptotic activity. Surprisingly, peak number IV showed the highest anti-apoptotic activity. In line with this, peaks I, III and IV were decreased when CM from SPIα cells labeled to equilibrium with [^{14}C]arachidonic acid were incubated with SPIβ cells for 1 h (Figure 3). This indicates that the labeled compounds in these peaks were bound or take up by the SPIβ cells.

Sample analysis by electrospray ionization mass spectrometry (ESI-MS) still showed a large number of different compounds (>30) present in the peak with the highest anti-apoptotic activity (IV). Further identification was hampered by lack of appropriate mass spectrometry knowledge on lipidomics and instability of the anti-apoptotic factors upon purification or extraction from the CM.

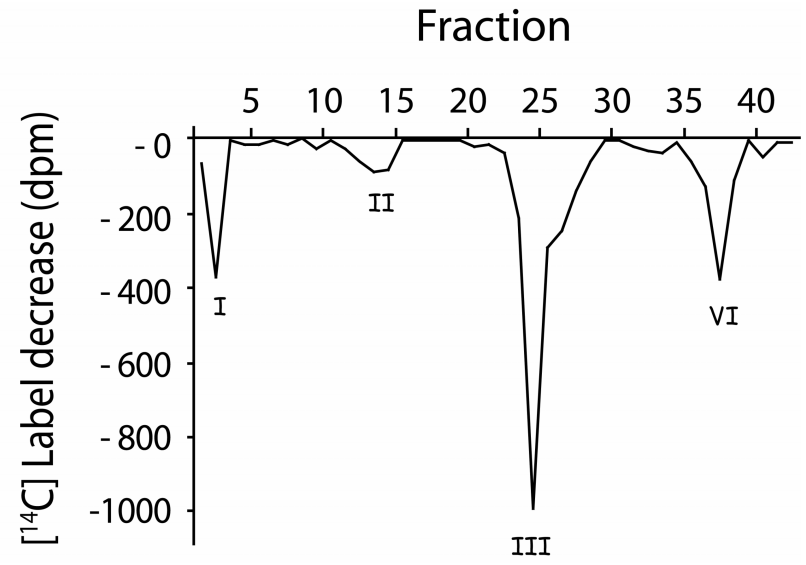

Figure 3. Decrease of [^{14}C] label in CM from SPIα cells upon incubation of SPIβ cells. CM was prepared from SPIα cells labeled to equilibrium with [^{14}C]arachidonic acid and split in 2 portions. SPIβ cells were incubated for 1 h with one portion of CM from SPIα cells. Both the incubated CM and the native CM were subjected to ethyl acetate extraction and separated by a C18 reversed phase column. The [^{14}C] label in the fractions collected from the reversed phase column was counted by liquid scintillation. The decrease in [^{14}C] label as a result of the incubation with SPIβ cells was calculated and illustrates the binding and/or uptake of [^{14}C] labeled compounds by SPIβ cells.

Endocannabinoids

Since CM from SPIα cells exhibit anti-apoptotic activity through activation of a cannabinoid 1-like (CB1) receptor [2], we investigated the involvement of endogenous cannabinoids 2-arachidonoylglycerol (2AG) and anandamide (AEA). Upon incubation of SPIβ cells with CM from SPIα cells labeled to equilibrium with sodium [^{14}C]acetate, radioactivity is taken up by the cells. The addition of CM from unlabeled SPIα cells competes with this uptake and reduced the uptake of radioactive label by 20% (Figure 4). Similarly, addition of SR141716A, a specific CB1 receptor antagonist, AEA (10 μM), arvanil (10 μM), 2AG (10 μM) and 2AG-ether (10 μM) reduced the uptake of radioactivity by 20%, 20%, 25%, 35% and 30% respectively. All endocannabinoids competed with the uptake in a concentration-dependent manner. In contrast, addition of unlabeled CM from wtNIH3T3 and SPIβ cells barely reduced the uptake of radioactive label by SPIβ cells. This strongly suggests that the [^{14}C] labeled CM from the SPIα cells contained compounds taken up by the CB1 and CB2 receptor.

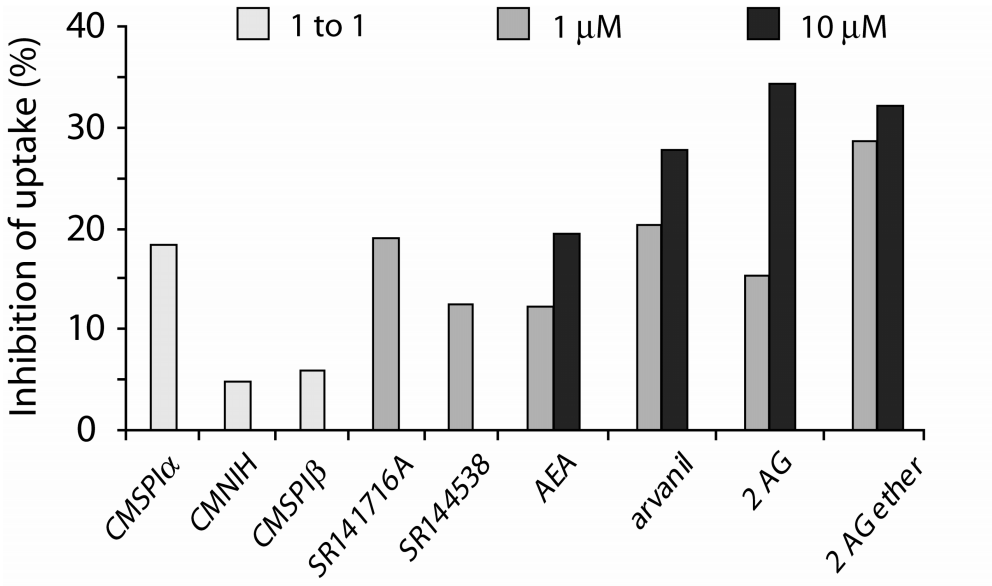

Figure 4. Competition for the uptake of [^{14}C] labeled compounds in CM from SPIα cells with CB1 agonists. After incubation for 2 h with DBB, the SPIβ cells were incubated with CM from SPIα cells labeled to equilibrium with [^{14}C]sodium acetate, which leads to uptake of the label by the SPIβ cells. The addition of unlabeled CM from SPIα cells, cannabinoid 1 (CB1) receptor antagonists and CB1 receptor agonist competes with the uptake of [^{14}C] label by SPIβ cells. The inhibitory effect of the CB1 receptor agonists was shown to be concentration-dependent.

Unexpectedly, HPLC-MS analysis of endocannabinoid levels showed that the level of 2AG was reduced in CM from SPIα cells compared with CM from wtNIH3T3 cells (9.5 ± 3.3 pmol/ml and 21.9 ± 1 pmol/ml, respectively; n = 3). In addition, free arachidonic acid decreased in CM from SPIα cells compared with CM from wtNIH3T3 (16.2 ± 0.7 pmol/ml and 58.6 ± 23.9 pmol/ml, respectively; n = 3) and no AEA or oleoylethanolamide (OEA) was detected in either CM from wtNIH3T3 or SPIα cells. Moreover, the level of 2AG in whole wtNIH3T3 and SPIα cells showed no significant difference (45.0 ± 16.5 pmol/mg of protein and 39.4 ± 12.2 pmol/mg of protein, respectively; n = 3). The incubation of SPIβ cells with 2AG or AEA (in DMEM containing 0.1% bovine serum albumin; DBB medium) showed that neither 2AG nor AEA displayed anti-apoptotic activity comparable with CM from SPIα cells thereby excluding the involvement of endocannabinoids 2AG and AEA (data not shown).

HPLC-MS

To gain further insight into the nature and identity of the secreted compounds, lipid extracts of CM from SPIα and wtNIH3T3 cells were compared by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and detected by mass spectrometry scanning between 250 m/z and 500 m/z. Comparison of CM extracted by methanol/chloroform (1:2, v/v) showed a substantial number of differences between CM from wtNIH3T3 cells (figure 5; panel A) and CM from SPIα cells (panel B). Using standards it was shown that arachidonic acid, 2AG and AEA eluted between 14 and 16 minutes and between 327 and 401 m/z, an area which displays a large number of PI-TPα-dependent differences. In contrast to the results obtained by ELISA [2], no prostaglandin E_2 (PGE_2) was detected in CM from wtNIH3T3 cells or in CM from SPIα cells. Furthermore, no PGE_2 ethanolamide and PGE_2 glycerol ester was detected.

The total mass spectra as a combination of the mass spectra of the HPLC fractions (Figure 5) are given in figure 6. The major m/z peaks are indicated. From visually comparing spectra ‘CM from SPIα’ and ‘CM from wtNIH3T3’ it is shown that associated with the overexpression of PI-TPα a major new peak is m/z 275, whereas

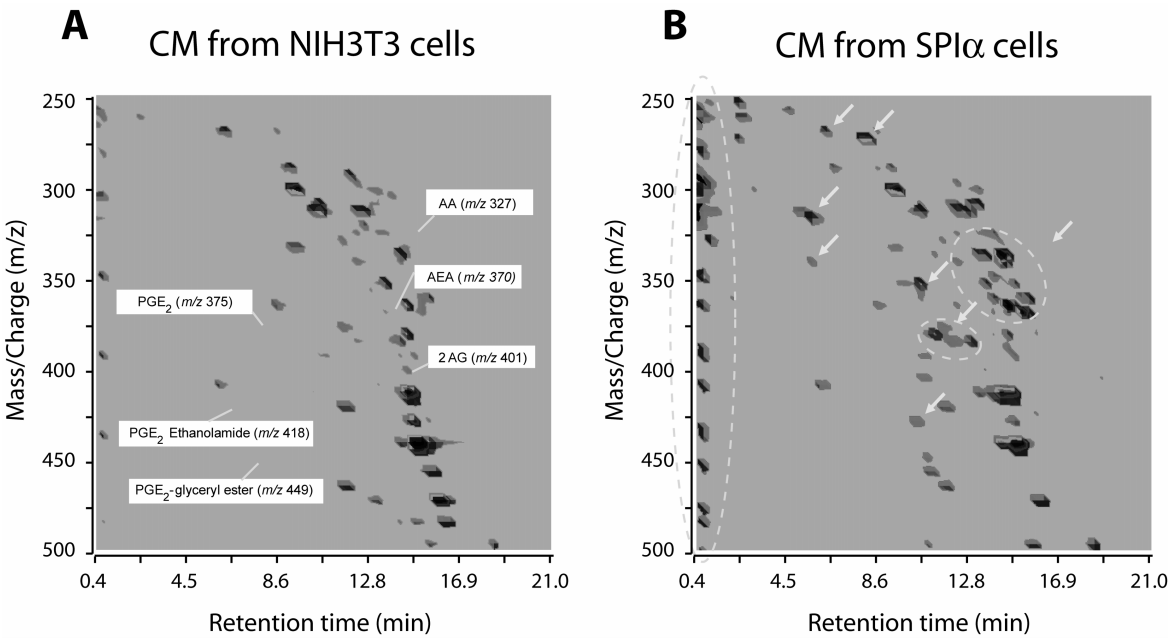

Figure 5. HPLC-MS analysis of CM from wtNIH3T3 cells and CM from SPIα cells. The CM from wtNIH3T3 cells and SPIα cells was subjected to methanol/chloroform extraction and analyzed by high-performance liquid chromatography (XDB Eclipse C18 column) coupled with mass spectrometry analysis. MS detection was set to scan from 250 m/z to 500 m/z in the positive ionization mode. Retention times of standards are indicated in the left panel. The arrows and circled zones in the right panel indicate the differences between CM from wtNIH3T3 cells and CM from SPIα cells.

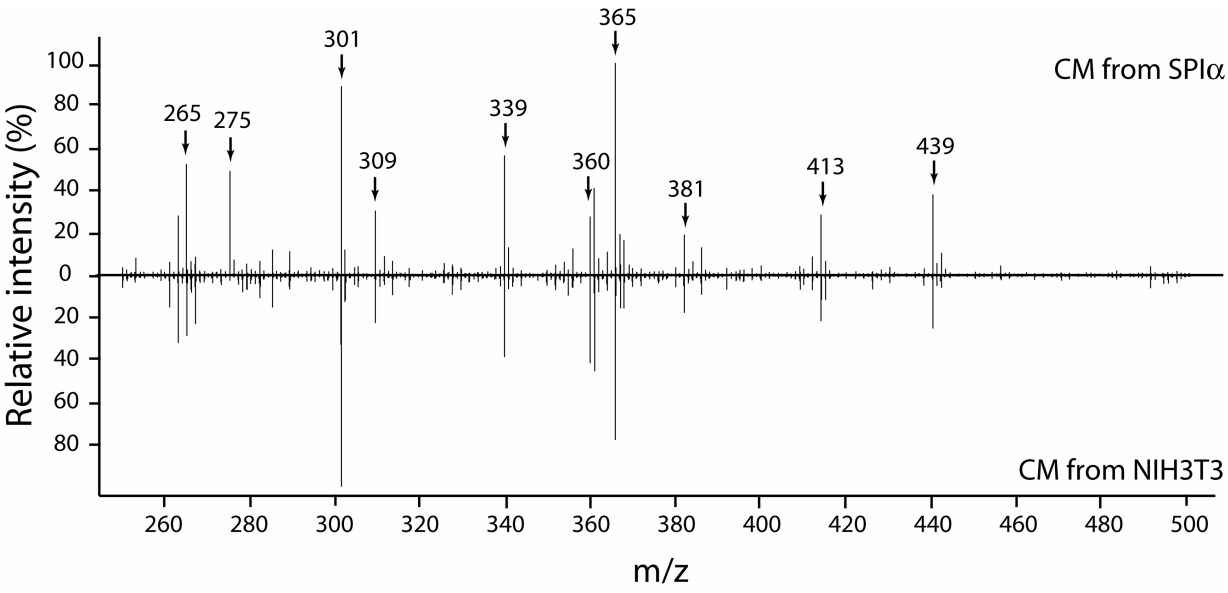

Figure 6. The total mass spectra as a combination of the mass spectra of the individual HPLC fractions. The conditioned media were subjected to methanol/chloroform extraction and analyzed by HPLC-MS as described in materials and methods. The total mass spectra was obtained by averaging the mass spectra of the individual fractions collected from the C18 reversed phase column between 0.4 min to 20 min.

peaks m/z 265, 309, 365, 413, and 439 are substantially increased. Other major peaks m/z 263 and 361 are not significantly changed. It should be noted that visual comparison of these spectra is restricted by the resolution of the figures.

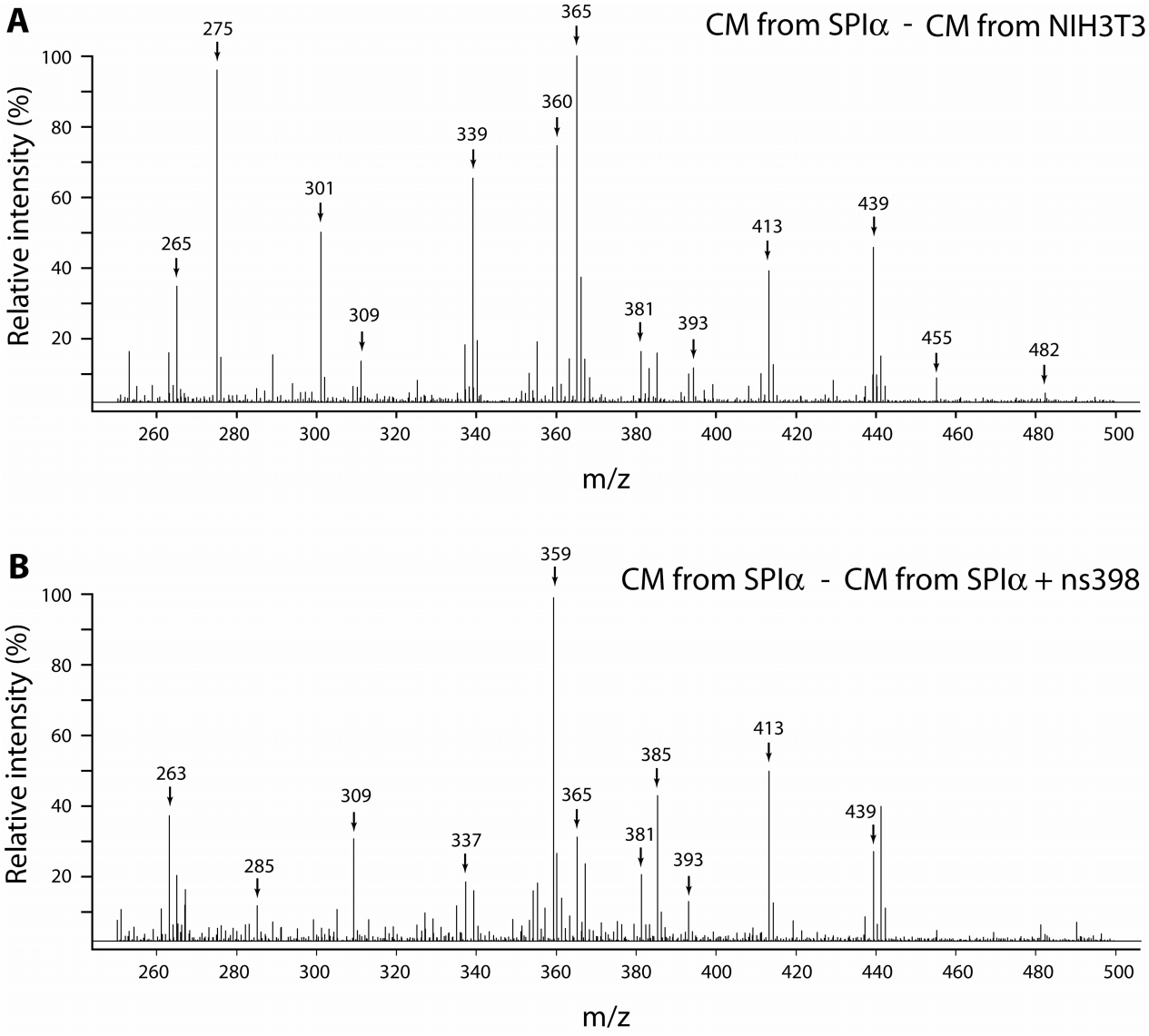

Figure 7. Quantitative subtraction of the mass spectra (from 250 m/z to 500 m/z). The conditioned media were subjected to methanol/chloroform extraction and analyzed by HPLC-MS as described in materials and methods. **A)** The total mass spectra of CM from wtNIH3T3 cells were subtracted from the total mass spectra of CM from SPIα cells revealing m/z values which are present in CM from SPIα cells, but not in CM from wtNIH3T3 cells or to a lesser extent. **B)** Total mass spectra of CM from SPIα cells produced in the presence of the COX-2 inhibitor NS398 was subtracted from the total mass spectra of CM from SPIα cells revealing compounds produced in a COX-2-dependent manner. Results were verified by a second method termed 'Compare LCMS' (ACD/MS Manager).

Quantitative subtracting (ACD/MS Manager; subtraction: m/z based; single peak only) of the total mass spectra of CM from wtNIH3T3 cells from the total mass spectra of CM from SPIα cells reveals the extra compounds (new or increased) secreted by SPIα cells expressed relative to m/z 365 (Figure 7A)(subtraction 1). The results from this subtraction method were verified by a second method termed 'Compare LCMS' (ACD/MS Manager). Furthermore, since the production of PI-TPα-dependent anti-apoptotic activity is partially dependent on cyclooxygenase 2 (COX-2), we looked for all the COX-2-dependent compounds secreted by SPIα cells. These compounds were identified by a second subtraction (Figure 7B)(subtraction 2) in which the total mass spectra of CM from SPIα cells produced in the presence of the COX-2 inhibitor NS398 was subtracted from the total mass spectra of CM from SPIα cells. The m/z values present in both subtraction 1 and 2 represent the extra COX-2-dependent compounds present in CM from SPIα cells compared with CM from wtNIH3T3 cells. These analyses were performed on eight different batches of conditioned medium. The PI-TPα- and COX-2-dependent peaks, which were common in the analysis of all batches represented about 70% of the total number of peaks recorded in any particular subtration. This leaves a list of relevant m/z values presented with the retention times (Figure 8 and table 2). The peak representing the most pronounced difference (365 m/z) was subjected to MS/MS analysis. However, the mass accuracy of the detector and the lack of a lipidomic MS/MS fragment database prevented identification.

Interestingly, upon incubation of SPIβ cells with CM from SPIα cells, compounds with m/z values 301, 337, 393 and 413 decreased in the CM. This may indicate that the SPIβ cells have preferentially take up some of the PI-TPα and COX-2-dependent eicosanoids (337, 393 and 413). It remains to be determined whether the actual anti-apoptotic factors are represented by these eicosanoids.

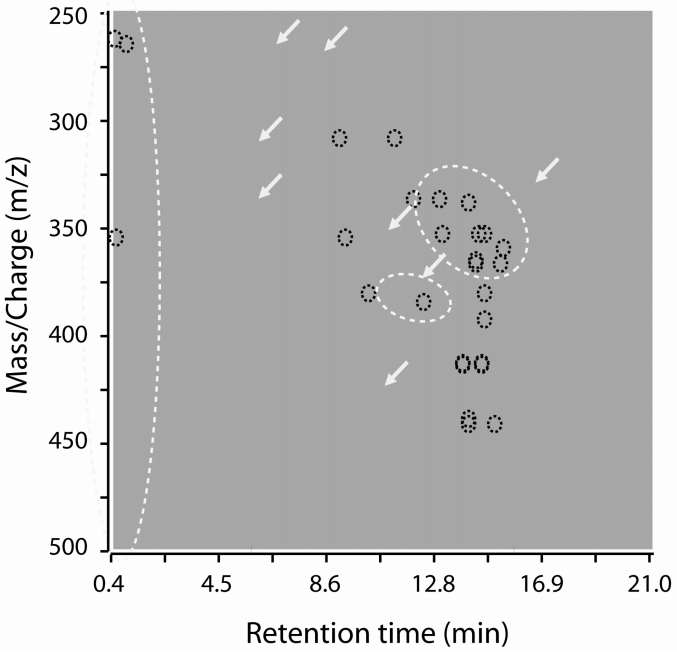

Table 2

m/z	Retention time (min)		
263	0.6		
265	1.1		
309	9.2	11.3	
337	12	13	
339	14.1		
353	13.1	14.5	14.7
355	0.7	9.4	
360	15.4		
365	14.4		
366	14.4		
367	14.4	15.3	
381	10.3	14.7	
385	12.4		
393	14.7		
413	13.9	14.6	
414	13.9	14.6	
439	14.1		
441	14.1	15.1	

Figure 8. Additional COX-2-dependent compounds present in CM from SPIα cells compared with CM from wtNIH3T3 cells. CM from wtNIH3T3 cells and SPIα cells were compared by HPLC-MS analysis. Quantitative subtraction of mass spectra resulted in a number of mass over charge values that represent the additional COX-2-dependent compounds present in CM from SPIα cells when compared with CM from wtNIH3T3 cells. The arrows in this figure are identical to the arrows in figure 4, illustrating that not all differences between CM from wtNIH3T3 cells and CM from SPIα cells are consistently different or COX-2-dependent.

Table 2. Mass over charge values that represent the additional COX-2-dependent compounds present in CM from SPIα cells when compared with CM from NIH3T3 cells.

Discussion

Previously we reported that mouse fibroblast cells with an increased expression of PI-TPα showed an increased production of lysoPI, glyceroPI, I(1)P, and I(2)P, which are all markers for enhanced degradation of PI by PLA and lysoPLA [2]. Since PI is highly enriched in arachidonic acid at the sn-2 position, the enhanced degradation of PI should result in an increase in free arachidonic acid. In contrast, the level of free (20:4) fatty acid decreased in CM from SPIα cells when compared with CM from wtNIH3T3 cells, suggesting that the released arachidonic acid is metabolized further. In line with this, we previously reported that SPIα cells produce and secrete COX-2-dependent mitogenic and

anti-apoptotic factors [2]. Analysis of CM from SPIα cells that was separated by gel filtration showed that a large portion of the anti-apoptotic factors is most likely bound by bovine serum albumin (BSA) present in the CM. Since BSA is a principal carrier of hydrophobic and negatively charged compounds like fatty acids, the suggestion arises that a large portion of the anti-apoptotic factors have properties similar to fatty acids. Increasing the stability of the anti-apoptotic factors seems to be an additional function of BSA since, in contrast to the neutral lipid extract of CM from SPIα cells, the native CM from SPIα cells is very stable during storage at 4^{o}C (unpublished data). Although a large fraction of the anti-apoptotic factors is most likely bound to BSA and therefore hydrophobic in nature, some arachidonic acid metabolites represented in peak 2 (figure 1) are revealed to be reasonably soluble in water (peak I and II; figure 2). Upon extraction with ethyl acetate and formic acid, which adjusts the pH of the CM to 2 [5], the anti-apoptotic activity present in CM from SPIα cells is retained in the organic phase. Since ethyl acetate only extracts neutral lipids, the anti-apoptotic factors are either neutral or acidic compounds at neutral pH. In addition, it was shown that the anti-apoptotic activity divides over three peaks when separated on a C18 reversed phase column. This strongly suggests that CM from SPIα cells contains more than one anti-apoptotic factor, with the bulk of the anti-apoptotic factors displaying very hydrophobic properties (similar or more hydrophobic than arachidonic acid). It is unclear whether the anti-apoptotic factors present in CM from SPIα cells exert their effect separately or in a synergistic manner. Furthermore, sample analysis by electron spray ionization mass spectrometry (ESI-MS) of peak IV, which contains an extremely hydrophobic arachidonic acid metabolite, still showed a large number of different compounds present in this peak.

We previously reported that the anti-apoptotic activity present in CM from SPIα cells activates a cannabinoid 1-like receptor [2]. Since the fraction containing the endocannabinoids (peak III; figure 2) showed some anti-apoptotic activity and was shown to be bound to or taken up by the SPIβ cells (figure 3), we further evaluated the involvement of endogenous cannabinoids and the cannabinoid 1 (CB1) receptor. All the endocannabinoids tested as well as a CB1 receptor antagonist competed with the uptake of radiolabelled compounds from CM from SPIα cells by SPIβ cells. This supports the proposed involvement of the CB1 receptor. On the other hand, the fact that (a) HPLC-MS

analysis showed that the level of 2AG decreased rather than increased in CM from SPIα cells compared with CM from wtNIH3T3 cells, (b) AEA was not present in either CM from SPIα cell or in CM from wtNIH3T3 cells, (c) no differences in 2AG and AEA levels were observed in SPIα and wtNIH3T3 cells and (d) 2AG and AEA displayed no anti-apoptotic activity comparable to CM from SPIα cells, excludes the involvement of these two endocannabinoids.

A different approach to identify the anti-apoptotic factors would be by identification of all differences between CM from wtNIH3T3 and CM from SPIα cells and testing these identified compounds for anti-apoptotic activity (separately or in combination). The comparison of CM from wtNIH3T3 cells and CM from SPIα cells by HPLC-MS showed a large number of PI-TPα-dependent differences between these media. Since only the COX-2-dependent anti-apoptotic activity seems to act via activation of a cannabinoid 1-like receptor (data not shown), we focused on the COX-2-dependent differences between CM from wtNIH3T3 and SPIα cells when compiling the list of possible candidates (Figure 8 and table 2). Figure 8 clearly shows that a large number of COX-2-dependent differences have hydrophobic properties similar to AA, 2AG and AEA. This suggests that it is probable that the anti-apoptotic factors are structurally closely related to these compounds. This is very striking since compounds that are metabolized by COX-2 usually become more water-soluble since double bonds are replaced by OH-groups. The compounds in table 2 that decrease upon incubation with SPIβ cells may be very promising (m/z 337, 393 and 413). Furthermore, it has become apparent that prostaglandin E_2 glycerol ester and prostaglandin E_2 ethanolamide are not involved since these compounds do not co-elute with any of the COX-2-dependent differences between CM from SPIα cells and CM from wtNIH3T3 cells. Prostaglandin glycerol esters and prostaglandin ethanolamides are produced when COX-2 utilize anandamide and 2-arachidonoylglycerol as a substrate [11-13]. Unfortunately, the mass accuracy of the mass spectrometer and -more importantly- the lack of a complete database for MS/MS analysis of lipid-fragments prevented further identification.

The possible role of PI-TPα in PLC-mediated inositol lipid signaling at the plasma membrane has been described in literature [14-16]. Moreover, the conversion of

diacylglycerol into 2AG by diacylglycerol lipase is well understood [17]. In previous studies we failed to observe differences in the levels of PI(4)P and PIP_2 in the SPIα cells [18]. The failure to detect differences in 2AG levels in SPIα cells and in CM from SPIα cells compared with wtNIH3T3 cells and CM from wtNIH3T3 cells respectively, illustrates once more that overexpression of PI-TPα does not affect PLC mediated inositol lipid signaling in wtNIH3T3 cells.

Ackowledgement

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Chapter 6

Summarizing discussion

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Phospholipid transfer proteins occur in a wide range of organisms, such as mammals, plants, yeast and fungi and are characterized by their ability to catalyze the transfer of phospholipids between membranes *in vitro* [1]. One of these proteins is the phosphatidylinositol transfer protein (PI-TP), which is highly conserved in mammalian tissues but also occurs in plants, yeast, insects and fungi strains [1, 2]. *In vitro* this protein is able to transfer phosphatidylinositol (PI) between membranes and to a lesser extent phosphatidylcholine (PC) [1, 3-5]. In mammals two soluble, low molecular weight isoforms have been identified (i.e. PI-TPα and PI-TPβ), the 271 amino acids residues of which are 77% identical and 94% similar [6, 7]. In addition, recently a novel PI-TPβ isoform with a naturally occurring Ser262Gln polymorphism was identified [8, 9]. Studies on mRNA and protein levels showed that PI-TPα and PI-TPβ are present in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas [10]. Despite the similarity between PI-TPα and PI-TPβ several differences have been revealed. In addition to being able to transfer PI and PC, PI-TPβ could also transfer sphingomyelin (SM), a property not displayed by PI-TPα [5]. This was recently confirmed by studies carried out by Morgan *et al.*, which have shown that the SM transfer activity depends on the C-terminal segment of PI-TPβ, which is substantially different from that of PI-TPα [9]. It was further shown that PI-TPα is mainly localized in the cytosol and the nucleus, whereas PI-TPβ is mainly associated with the Golgi complex [5, 8, 9, 11].

PI-TP gene ablation and natural mutants in which PI-TPα was greatly reduced underline the critical functions of these proteins. Specifically PI-TPβ gene ablation failed to give viable embryonic stem cells [12], whereas PI-TPα gene ablation did not interfere with embryonic development. However, in the latter case the PI-TPα$^{-/-}$ pups suffered an early juvenile death upon birth. Vibrator mice which have a 80% reduced level of PI-TPα, display an early onset of progressive action tremor and degeneration of brain stem and spinal cord neurons [13]. Similarly, ablation of PI-TPα function in PI-TPα$^{-/-}$ mice resulted in spinocerebellar disease characteristics, hypoglycemia, and intestinal steatosis [14]. These latter studies fail to give a clue as to why the absence of PI-TPα or its

severally reduced levels is accompanied by these very grave physiological disorders. On the other hand, increased expression of PI-TPα in mouse fibroblast cells (SPIα cells) leads to a significantly increased rate of proliferation indicating that PI-TPα may be involved in the production of a mitogenic factor [15]. In line with this, WRK-1 rat mammary tumor cells which as a result of antisense transfection expressed approximately 25% less PI-TPα than control clones showed a decreased growth rate [16]. In contrast to PI-TPα, overexpression of PI-TPβ in fibroblast cells diminished the rate of proliferation [17].

PI-TPα and phospholipase A activity

In SPIα cells the levels of lysoPI, glycerophosphoinositol, inositol-1-P and inositol-2-P are increased indicating that a phospholipase A (PLA) with affinity for PI is activated [18]. Since PI is highly enriched in arachidonic acid, one may presume that a significant amount of this fatty acid is produced. Although double-labeling experiments with [^{3}H]-*myo*-inositol and [^{14}C]arachidonic acid have been performed in SPIα cells, it remained unresolved whether PLA_1 or PLA_2 was constitutively activated. The inositol phosphate derivatives identified (i.e. lysoPI and glycerophosphoinositol) fail to indicate whether PI was degraded by PLA_1 and subsequently PLA_2 or vice versa [19-21]. In both situations lysoPI and free arachidonic acid are produced. Under these conditions arachidonic acid is converted into arachidonic acid metabolites among which prostaglandins, leukotrienes and thromboxanes [22]. The mechanism by which the increased level of PI-TPα in SPIα cells results in increased PLA activity and the subsequent degradation of PI into lysoPI in these cells is still unclear [15]. Since lipid hydrolysis by PLA_2 proceeds more efficiently in a membrane where the lipid substrate is made more accessible [23, 24], it is possible that PI-TPα assists in PI hydrolysis by extracting a PI-molecule from the membrane and by presenting it directly to PLA. On the other hand, it has been suggested that increased levels of PI-TPα could lead to a surplus of PI at certain sites of the cell, which would then be corrected by PLA-mediated degradation. Another possibility is that PI-TPα acts as a lipid sensor and that increased levels of PI-TPα carrying a PI-molecule directly

affect PLA activity comparable to Sec14p and the Nir/rdgB family members controlling PC synthesis (see below). The specific relationship between PI-TPα and PLA is underlined by the fact that fibroblast cells overexpressing PI-TPβ (SPIβ cells) have a the PI-turnover comparable to that of wtNIH3T3 cells [15].

Effect of PI-TPα on apoptosis

In **chapter 2** we show that SPIα cells produce and secrete a mitogenic factor. In addition, we show that SPIα cells are extremely resistant towards UV-induced apoptosis when compared with wild-type NIH3T3 (wtNIH3T3) cells. The incubation of wtNIH3T3 or SPIβ cells with conditioned medium (CM) from SPIα cells induces resistance of these cells against UV-induced apoptosis. Preliminary experiments with wtNIH3T3 cells in which the level of PI-TPα was reduced by small interference RNA, showed that the anti-apoptotic activity present in the CM from these cells was diminished. At this stage it is not clear whether the decline in anti-apoptotic activity is a direct result of either the reduction of PI-TPα levels or the reduced viability of these treated cells (preliminary data not shown).

Since incubation of the SPIα cells with a cyclooxygenase-2 (COX-2) inhibitor reduces the anti-apoptotic activity of the ensuing CM, it is inferred that the active factor(s) is a COX-2-dependent arachidonic acid metabolite. It is well established that COX-2 plays an important role in cell growth and cell survival by generating anti-apoptotic eicosanoids from pro-apoptotic arachidonic acid [25-28]. In this process the release of arachidonic acid by PLA_2 is the rate limiting step [29]. Compared with wtNIH3T3 cells, SPIα cells contain increased levels of COX-1 and COX-2. In agreement, CM from SPIα cells is highly enriched in arachidonic acid metabolites (e.g. 0.5 ng/ml PGE_2 in CM from SPIα cells compared with 0.1 ng/ml in CM from wtNIH3T3 cells) as determined by an ELISA. In contrast to what we expected, PGE_2 with or without $PGF_{2\alpha}$ showed no growth-promoting effect on wtNIH3T3 cells. However, $PGE_2/PGF_{2\alpha}$ enhanced to some extent the survival of wtNIH3T3 cells after UV irradiation. Incubation of SPIβ cells with $PGE_2/PGF_{2\alpha}$ had no effect either on growth rate or survival (chapter 4).

This strongly suggests that the anti-apoptotic activity observed is due to an as yet unidentified arachidonic acid metabolite.

A particular group of eicosanoids, the endocannabinoids, have been shown to inhibit neurodegeneration in rat brain by activation of the cannabinoid 1 (CB1) receptor [30]. Since PI-TPα seems to be involved in maintaining neural integrity in the vibrator and PI-TPα$^{-/-}$ mice, we investigated whether the anti-apoptotic activity acts through activation of the CB1 receptor. The anti-apoptotic activity of CM from SPIα cells was reduced by inhibitors of G protein-coupled receptors as well as by a specific antagonist of the CB1 receptor. The involvement of the CB2 receptor could not be excluded. This inhibitory effect was restricted to the COX-2-dependent anti-apoptotic activity since the remaining anti-apoptotic activity present in CM prepared in the presence of a COX-2 inhibitor could not be inhibited by a G protein-coupled receptor inhibitor or by a specific antagonist of the CB1 receptor (data not shown). The activation of the CB1 receptor suggests that the anti-apoptotic factor(s) present in CM from SPIα cells could be a member of the endocannabinoid family. Endocannabinoids are known to have a dual role in the regulation of apoptosis [31, 32]. Some studies report that cannabinoids induce growth arrest or apoptosis in transformed cells. On the other hand, the majority of the studies show that cannabinoids protect cells from apoptosis (reviewed in [33]).

Signaling pathways affected by the PI-TPα-dependent survival factor(s)

In **chapter 3** the pathways by which the PI-TPα-dependent survival factor(s) affects cell survival are explored. Since the discovery that cannabinoids bind to specific receptors in the brain, the signaling mechanisms triggered by these activated receptors have been studied extensively. To date, two types of cannabinoid receptor are known, which have a different tissue distribution. The CB1 receptor is expressed in central nervous system areas which are associated with motor coordination, learning, memory and higher cognitive functions such as cerebral cortex, hippocampus, cerebellum and basal ganglia [34]. The CB2 receptor is predominantly localized in cells belonging to the immune system [35, 36]. The CB receptors are coupled to $G_{i/o}$ proteins and are shown to modulate adenylate cyclase, ion channels, hydrolysis of sphingomyelin, generation of

ceramide, activation of phospholipases, regulation of nitric oxide species and extracellular signal-regulated kinase cascades like mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3-K) and focal adhesion kinase (FAK) [37-39]. The p42/p44 MAPK pathway and the PI3-K-Akt/PKB pathway are well known in linking surface receptor activation to cell survival [40-44] [45]. It has been reported that the p42/p44 MAPK pathway affects apoptosis by promoting expression of IAP (inhibitor of apoptosis proteins) [46-48]. Upon activation, the Akt/PKB pathway has a direct effect on cell survival by inhibiting the pro-apoptotic Bcl-2 related protein, BAD and by inhibiting caspases 9. Furthermore, Akt/PKB affects apoptosis on a transcriptional level both by activation of NF-κB and CREB, which regulate the transcription of pro-survival genes like Bcl-xL, caspases inhibitors and by inhibition of YAK and Forkhead, which regulate the transcription of pro-apoptotic genes like JNK and Bax [49-53].

Comparison of CM from wtNIH3T3 cells with CM from SPIα cells revealed that the PI-TPα-dependent anti-apoptotic factors act via both the p42/p44 MAPK pathway and the Akt/PKB pathway, thereby activating the anti-apoptotic transcriptional factor NF-κB. In addition to activating the p42/p44 MAPK and Akt/PKB pathways, the activation of the CB1 receptor can affect cell survival by modulating the cAMP/protein kinase A (PKA) pathway or SM hydrolysis and ceramide formation [54, 55]. The role of the cAMP/PKA pathway and ceramide in apoptosis has been the focus of many studies. In general, the upregulation of the cAMP/PKA pathway inhibits proliferation and results in apoptosis [56] and the increase in intracellular ceramide levels induces apoptosis and reduces cell proliferation [57]. We have found that the survival of SPIβ by CM from SPIα cells in the presence of a specific inhibitor of PKA did not further increase (data not shown). Furthermore, analysis of lipid extracts of cells labeled with L-3-[^{14}C]serine and incubated with CM from SPIα cells showed no significant differences in SM, ceramide, sphingosine and sphingosine 1-P compared with cells incubated with DBB. However, it can not be excluded that modulation of the cAMP/PKA or ceramide pathway pathways plays a role in the protective effect of CM from SPIα cells. In addition, since the p42/p44 MAPK and Akt/PKB pathways also affect transcriptional factors other than NF-κB, it is most likely that the activation or inhibition of additional transcription factors like CREB,

YAK and Forkhead is involved in the mitogenic and anti-apoptotic effect of the PI-TPα-dependent factors.

Furthermore, we demonstrated that the cellular effect of the anti-apoptotic factor is independent of PLC activation since the addition of CM from SPIα cells had no effect on the translocation of YFP-tagged C2 domain from PKC (Chapter 3; figure 6). This is in line with the fact that CB1 receptor signaling leads to the activation of the G protein $G_{i\alpha}$ which does not activate PLCβ [35, 58-60]. This observation does not necessarily contradict with several studies showing a relationship between PI-TPα and the GPCR-mediated activation of phospholipase C (PLC) upon addition of PI-TPα to cytosol depleted semi-intact cells [61-63]. In the latter case, it is proposed that PI-TPα is essential for enhanced formation of PI(4)P and $PI(4,5)P_2$ as substrates of PLC. On the other hand, exploring the *in vivo* function of PI-TPα in intact cells could not confirm the proposed role of PI-TPα in phospholipase C-mediated inositol lipid signaling since no changes were found in PI(4)P, $PI(4,5)P_2$ and IP_3 levels upon overexpression of PI-TPα [15]. Moreover, since 2AG is synthesized via the conversion of diacylglycerol (generated via the degradation of PIP_2 by PLC) by diacylglycerol lipase [64], the failure to detect differences in 2AG levels in SPIα cells and in CM from SPIα cells compared with wtNIH3T3 cells and CM from wtNIH3T3 cells respectively, illustrates once more that overexpression of PI-TPα does not affect PLC-mediated inositol lipid signaling in wtNIH3T3 cells (Chapter 5). In addition, failure to detect alterations in the bulk of PI, PI(3)P, PI(4)P or $PI(4,5)P_2$ levels in PI-TPα$^{-/-}$ murine embryonic stem cells or murine embryonic fibroblasts confirmed that the role of PI-TPα in PLC-mediated inositol lipid signaling may be very limited in intact cell [12, 14].

Effect of PI-TPβ on apoptosis

Since PI-TPα and PI-TPβ show a high amino acid sequence homology and since both proteins transfer PI and PC between membranes, their functions were expected to be very similar. Nevertheless, the cellular function of PI-TPβ appears to be completely different from that of PI-TPα. Whereas SPIα cells demonstrate an increased rate of proliferation

and resistance to apoptosis, it has been shown that SPIβ cells have a decreased growth rate and a lower resistance against UV-induced apoptosis [17, 65]. In **chapter 4** we describe several observations that may explain why SPIβ cells are more susceptible to the induction of apoptosis, not only by UV irradiation but also by tumor necrosis factor α or serum starvation. In contrast to SPIβS262A (wild-type NIH3T3 cells overexpressing a mutant PI-TPβS262A lacking the protein kinase C-dependent phosphorylation site) and wtNIH3T3 cells, SPIβ cells maintain the total level of SM under conditions where SM is degraded in the plasma membrane to ceramide by exogenous sphingomyelinase [4, 65]. Although increased levels of PI-TPβ appear to be required for SPIβ cells to rapidly convert ceramide to SM, we found that steady-state levels of ceramide in SPIβ and wtNIH3T3 cells are similar. However, when investigating the acyl chain composition of SM and ceramide in SPIβ cells, we observed that the molecular species of the fatty acids of SM and ceramide are different. SPIβ cells appear to have relatively less short chain (C16) and more long chain species (C24:1/0) compared with wtNIH3T3 cells. Although the mechanism by which PI-TPβ facilitates the shift from short chain to long chain ceramide/SM species is not known, it has been reported that enhanced levels of intracellular C22 and C24 ceramide leads to apoptosis in osteoblast cells [66]. In addition, several reports show that that apoptotic stimuli including TNFα [67], ionizing radiation [68] and B-cell receptor cross-linking [69] can generate ceramide by the induction of SM hydrolysis through the action of sphingomyelinases or the *de novo* pathway. The shift towards relatively more long chain ceramide species in SPIβ cells could render the SPIβ cells more sensitive to the induction of apoptosis. In addition, it could be possible that the relative enrichment of long chain SM species in SPIβ cells has an effect on plasma membrane properties affecting the activity or susceptibility of transmembrane receptors involved in growth and survival [70]. In addition, the shift in SM species may possibly have an adverse effect on the presence of receptors involved in anti-apoptotic pathways as illustrated by the reduced level of the CB1 receptor in the plasma membrane of SPIβ cells [71]. It remains to be established whether a reduction in the level of E-prostanoid (EP) receptors is involved in the lack of COX-2 upregulation by prostaglandin (PG) E_2 and $PGF_{2\alpha}$ in SPIβ cells. In general, reduced COX-2 expression

enhances apoptosis and more specifically reduces the incidence and progression of tumors in animal models [25, 72-74]. The fact that PGE_2 and $PGF_{2\alpha}$ are unable to upregulate COX-2 and unable to activate p42/p44 MAP kinase in SPIβ cells coincides with the failure of these prostaglandins to protect SPIβ cells against UV-induced apoptosis. In contrast, incubation with these prostaglandins upregulates the expression of COX-2 and activates the p42/p44 MAP kinase pathway in wtNIH3T3 and SPIβS262A cells, thereby protecting these cells against UV-induced apoptosis.

Since SPIβ cells contain about equal levels of PI-TPα compared with wtNIH3T3 cells, hence producing equal amounts of PI-TPα-dependent anti-apoptotic factors, the apparent lack of anti-apoptotic activity in CM from SPIβ cells was surprising. However after heat denaturation, an anti-apoptotic activity comparable to that of CM from wtNIH3T3 cells and CM from SPIβS262A cells was recovered. Moreover, in line with the lack of anti-apoptotic activity, CM from SPIβ cells does not induce the expression of COX-2 or the activation of p42/p44 MAP kinase. In contrast, CM from wtNIH3T3, SPIβS262A cells or heat-treated CM from SPIβ cells does induce the expression or activation of these anti-apoptotic markers in SPIβ cells.

Although recent reports question the role of the Ser-262 phosphorylation-site in PI-TPβ in the localization of this protein to the Golgi system [4, 8, 9], it is clear that the phosphorylation of the Ser-262 residue is critical for the apoptosis sensitivity of the SPIβ cells [17]. Namely, when the PKC-dependent phosphorylation site, Ser-262, is exchanged with an alanine or aspargine, the mutant SPIβ cells express signaling pathways involved in apoptosis and survival comparable with wtNIH3T3 cells. However, mutation of Ser-262 does not affect the *in vitro* transfer activity [4]. This illustrates the critical role of the phosphorylation of Ser-262 in PI-TPβ in the regulation of apoptosis.

Characteristics of the anti-apoptotic factor(s)

In **chapter 5** characteristics of the anti-apoptotic factors present in CM from SPIα cells are described and a class of likely candidates is identified. As previously mentioned, the enhanced degradation of PI into lysoPI and glycerophosphoinositol and the

involvement of COX-2, suggest that the anti-apoptotic factors could be arachidonic acid metabolites. The fact that the major fraction of the anti-apoptotic activity appears to be bound to bovine serum albumin (BSA), supports the proposed fatty acid nature of the factor(s). Besides binding the anti-apoptotic factor(s) upon secretion by SPIα cells, BSA seems to play an additional role in increasing the stability the factor(s) since, in contrast to the neutral lipid extract of CM from SPIα cells, the native CM from SPIα cells is very stable during storage at 4^{o}C.

Acidic as well as neutral compounds are extracted from the medium by a neutral lipid extraction method when the acidity of the medium is adjusted to pH 2. The fact that the organic phase retains all the anti-apoptotic activity upon neutral lipid extraction by ethyl acetate, suggests that the anti-apoptotic factor(s) is either neutral or negatively charged at pH 7.

In addition, it was shown that the anti-apoptotic activity distributes over three peaks when the neutral lipid extract of CM from SPIα cells is separated on a C18 reversed phase column. This means that CM from SPIα cells contains more than one anti-apoptotic factor, with the bulk of the anti-apoptotic factors displaying very hydrophobic properties (similar or more hydrophobic than arachidonic acid). It is unclear whether the anti-apoptotic factors present in CM from SPIα cells exert their effect separately or in a synergistic manner.

We previously reported that the anti-apoptotic activity present in CM from SPIα cells activates a cannabinoid 1-like receptor [75]. Although all endocannabinoids tested interfered with the uptake of radiolabelled compounds from CM from SPIα cells (chapter 5; figure 4), the involvement of 2-arachidonyl glycerol (2AG) and anandamide (AEA) is excluded since no increase in these endocannabinoids are observed in either the SPIα cells or in CM from SPIα cells when compared with wtNIH3T3 cells and CM from wtNIH3T3 cells. On the other hand, the fact that (a) a large number of COX-2-dependent differences between CM from SPIα cells and CM from wtNIH3T3 cells have hydrophobic properties and masses similar to AA, 2AG and AEA (chapter 5, figure 7), (b) endocannabinoids interfere with the uptake of radiolabelled compounds from CM from SPIα cells, and (c) the anti-apoptotic activity present in CM from SPIα cells can be

inhibited by a specific antagonist for the CB1 receptor (chapter 2, figure 7), strongly suggests that the some of the anti-apoptotic factors secreted by SPIα cells belong to the family of endocannabinoids. Further analysis was hampered by instability of the anti-apoptotic factors upon extraction and because of the lack of a database for lipid fragments.

Surprisingly, analyzing CM from SPIα cells subjected to methanol/chloroform extraction by LC/MS did not confirm the presence of prostaglandin E_2, the presence of which was indicated by the ELISA and 1-dimensional thin layer chromatography (chapter 2). It has been reported that prostaglandins may be misidentified by ELISA since several commercial antibodies raised against prostaglandins show major cross-reactivity with endocannabinoids or prostaglandin-like metabolites like prostaglandin E_2 glycerol ester and prostaglandin E_2 ethanolamide [76]. Both an inappropriate extraction method prior to LC/MS analysis and the misidentification of prostaglandins by ELISA could explain this discrepancy. On the other hand, no prostaglandin E_2 glycerol ester and prostaglandin E_2 ethanolamide was detected in CM from SPIα cells.

PI-TP related proteins

Since the discovery of phospholipid transfer proteins, a number of related proteins have been discovered which resemble PI-TPα and PI-TPβ either in amino acid sequence or in *in vitro* transfer activity. The most prominent are Sec14p, the 38-kDa human retinal degeneration Bb (MrdgBβ) protein and the high molecular weight, membrane associated Nir/RdgB family.

Nir/RdgB family

The first member of the Nir/RdgB family was detected in *Drosophila*, denoted as DrdgB and is primarily expressed in the retina [77]. DrdgB is a 160 kDa, membrane-bound protein, which contains an acidic, calcium-binding domain, six putative membrane-spanning regions, and a carboxyl-terminal domain. The amino-terminal 281 amino acids consist of a PI-transfer domain approximately 40% identical with PI-TPα. In

line with this, it has been reported that DrdgB is able to transfer PI and PC *in vitro*, which suggests that DrdgB is involved in phospholipid transport, metabolism and signaling [77]. Since DrdgB$^{-/-}$ flies suffer from light-induced retinal degeneration, DrdgB was implicated in the fly phototransduction cascade, a process in which the PI-TP-domain plays a critical role [78]. On the other hand, swapping the PI-TP-domain of DrdgB with PI-TPα resulted in a nonfunctional DrdgB chimera, which suggests that the phospholipid binding/transfer activity of the DrdgB-PI-TP domain is not the sole functional activity of this domain [78]. More recently, rdgB homologues have been identified in other species, i.*e.* worms, fishes and mammals [79-82]. The mammalian homologues Nir2 (also known as M-RdgB1, M-rdgBα and PITPnmα) and Nir3 (also known as M-RdgB2 and PITPnmβ) consist of an amino-terminal PI-TP-domain and a highly conserved carboxy-terminal section containing the tyrosine kinase Pyk2-binding domain [83]. The PI-TP domains of Nir2 and Nir3 are 45% identical to PI-TPα, 72% identical with each other and 65% identical with the *Drosophila* homologue. Nir2 and Nir3 are uniformly expressed in all classes of retinal neuronal cells. Similar to the Drosophila homologue DrdgB, the Nir proteins are involved in the regulation of lipid trafficking, metabolism and signaling in processes like cell morphogenesis and cytokinesis [84]. More specifically, it is proposed that Nir2 is involved in the regulation of $PI(4,5)P_2$ by a direct interaction with PI 4-kinase, which phosphorylates PI to produce PI(4)P. Subsequent phosphorylation by PI 5-kinase results in the production of $PI(4,5)P_2$, a lipid shown to be important for cytokinesis. Moreover, Nir2 was shown to be critical for controlling protein transport from the trans-Golgi network to the plasma membrane by maintaining the diacylglycerol level in the Golgi system [85]. In this process, Nir2 functions as a lipid-sensor, which regulates the diacylglycerol level by controlling the PC synthesis through the CDP-choline pathway, hence controlling diacylglycerol consumption.

Sec14p

Sec14p, the PI-TP homologue in yeast is primarily cytosolic [86], but has also been observed associated with the Golgi system, where it is supposed to be a major regulator of phosphatidylcholine (PC) homeostasis in yeast [87]. It was hypothesized that, when

PC levels increase in the Golgi membrane Sec14p will bind PC. Similar to Nir2, it has been shown that the PC-bound form of Sec14p inhibits PC synthesis via the CDP-choline pathway, thereby decreasing the DAG consumption in this pathway. Upon decrease in PC levels, the amount of Sec14p bound to PC is reduced, resulting in a concomitant increase in the level of PC synthesis. Since vesicle formation from the trans-Golgi is positively modulated by DAG and negatively by PC, Sec14p possibly regulates protein transport from the trans-Golgi to the plasma membrane by controlling PC levels [87]. On the other hand, recent reports show that the *in vitro* PC transfer activity of Sec14p is not required for its function, since yeast carrying a Sec14p mutant not capable of PC transfer *in vitro* was viable with improved secretory activity compared with a sec14p-deficient yeast strain [88].

Concluding remarks

Several studies investigating the *in vivo* function of PI-TPα could not confirm the role of PI-TPα in PLC-mediated lipid signaling currently reported in literature. Since neither overexpression nor knockout of PI-TPα showed any changes in PLC-mediated inositide lipid signaling we conclude that PI-TPα has a different *in vivo* function. Studies using vibrator mice and PI-TPα$^{-/-}$ mice have shown a role for PI-TPα in maintaining neural integrity in these mice. Given that PI-TP transfer activity is particularly high in synaptosome and myelin fractions from rat brain as a result of high levels of PI-TPα [17, 89], we propose that the neuronal cells of mice may be well protected against apoptosis by the production of anti-apoptotic factors mediated by PI-TPα. In line with this, it has recently been shown that the PI-TPα-dependent anti-apoptotic factors are able to protect cultured primary neurons against serum deprivation induced cell death [90]. The apparent lack for the requirement of PI-TPα for embryonic development, since PI-TPα$^{-/-}$ mice develop to term and are phenotypically normal, can be explained by a maternal supply of the anti-apoptotic factors.

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NL

Samenvatting

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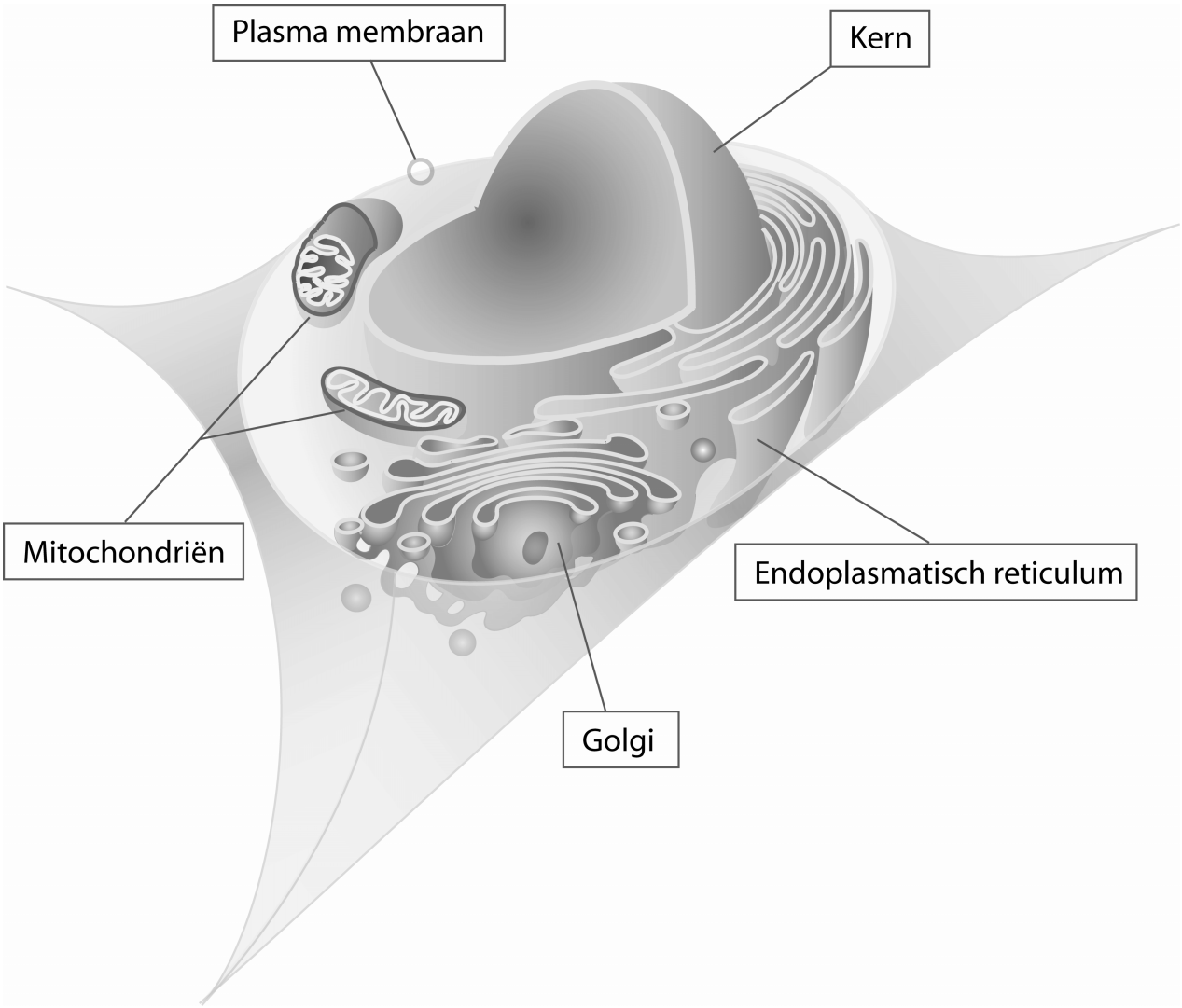
De eerste levende cel is waarschijnlijk rond 3,5 miljard jaar geleden op de aarde ontstaan door spontane reacties van moleculen in een chemisch labiel milieu. Een cel is de kleinste functionele eenheid waaruit alle organismen zijn opgebouwd. Tegenwoordig bestaan er organismen die uit vele miljarden cellen zijn opgebouwd. Cellen worden van hun omgeving gescheiden door een plasma membraan dat als een waterdichte barrière dient. Eén van de belangrijkste bouwstenen van een membraan zijn lipiden. Lipiden bestaan uit hydrofobe staarten (vetten) en een hydrofiele groep en komen in veel verschillende vormen voor, zoals ondermeer fosfolipiden (vetten + fosfaatgroep), glycolipiden (vetten + suikergroepen) en cholesterol (vetachtige stof). Een tweede belangrijke bouwsteen voor membranen zijn eiwitten (ook wel proteïne genoemd). Eiwitten zijn grote moleculen die bestaan uit een lange keten van diverse aminozuren en kunnen zowel in een membraan als vrij in het cytosol (interne vloeistof van een cel) voorkomen. In het plasma membraan zijn eiwitten verantwoordelijk voor selectief doorlaten van moleculen (kanalen) of doorgeven van signalen over het membraan (receptoren). Ook in de cel zijn er processen die door middel van een membraan van elkaar gescheiden moeten worden. Hierdoor ontstaan afzonderlijke compartimenten die organellen worden genoemd. Elk compartiment heeft een aparte functie. De kern (καρυον = noot of kern in het Grieks) bevat bijvoorbeeld het grootste deel van het DNA, dat drager is van de genetische informatie (figuur 1). Andere voorbeelden zijn de mitochondriën (μιτο = schroefdraad en χονδριον = korreltje in het Grieks), die de energie, aanwezig in suikers en vetten, omzetten en ter beschikking stellen aan energievragende processen in de cel. Het endoplasmatisch reticulum (reticulum = netwerk in het Latijn), dat zorg draagt voor transport van verschillende stoffen en de productie van vetten en bepaalde eiwitten. En het Golgi apparaat (ontdekt door Camillo Golgi), met als primaire functie het modificeren en sorteren van bepaalde lipiden en eiwitten en het vervolgens transporteren naar de plaats van bestemming.

Vanwege het hydrofobe karakter (slecht wateroplosbaar) van lipiden komen ze niet gemakkelijk uit een membraan. Daarom zijn er eiwitten nodig die lipiden uit een membraan kunnen opnemen om deze vervolgens te transporteren of te laten modificeren.



Figuur 1. Schematische weergave van een dierlijke cel.

Fosfatidylinositol transport eiwit (PI-TP) behoort tot een groep van eiwitten die fosfolipiden kunnen binden en transporteren tussen membranen. PI-TP's zijn gedurende de evolutie weinig veranderd en komen in veel verschillende organismen voor zoals zoogdieren, vogels, planten, insecten en verschillende soorten gist en schimmels. In zoogdieren zijn twee PI-TP's geïsoleerd en geïdentificeerd, PI-TPα en PI-TPβ. Deze isovormen kunnen allebei het lipide fosfatidylinositol (PI) en, in mindere mate, het lipide fosfatidylcholine (PC) transporteren *in vitro*. Daarnaast kan PI-TPβ ook het lipide sfingomyeline (SM) transporteren. Een tweede belangrijk verschil is de plaats waar deze

eiwitten voorkomen in een cel. PI-TPα is voornamelijk in de kern en het cytoplasma aanwezig, terwijl PI-TPβ hoofdzakelijk aan het Golgi apparaat gebonden is. Het verschil in lokalisatie van beide PI-TP's suggereert dat beide PI-TP's een aparte functie hebben. Het belang van deze eiwitten in een organisme blijkt uit het feit dat muizen embryo's zonder PI-TPβ ver voor de geboorte sterven. Muizen embryo's zonder PI-TPα worden wel "gezond" geboren, maar sterven binnen 14 dagen door het afsterven van neuronen (cellen die signalen vanuit de hersenen doorgeven aan spieren).

Naast dat fosfolipiden belangrijke bouwstenen zijn voor een membraan, kunnen ze ook omgezet worden naar moleculen die specifieke signalen doorgeven. In sommige gevallen wordt het totale lipide aangepast (bijv. PI krijgt extra fosfaatgroepen (Addendum, figuur 3, pad II)), in andere gevallen worden moleculen van het lipide afgeknipt (bijv. PI verliest een staart en de fosfatidylinositol groep bij de productie van een endogeen cannabinoid (Addendum, figuur 3, pad II)). In weer andere gevallen wordt één van de (vetzure)staarten van het lipide afgeknipt, waarna dit vetzuur wordt verwerkt tot een signaalmolecuul (prostaglandines, tromboxanen, prostacyclines). Signaalmoleculen kunnen er onder andere voor zorgen dat een cel gaat delen/groeien, maar ook dat een cel dood gaat en netjes wordt opgeruimd door het organisme. Dit laatste proces heet apoptose (ook wel geprogrammeerde celdood genoemd) en komt van het Griekse woord απόπτόσισ, wat "vallen" betekent, gelijk aan een blad dat in de herfst van een boom valt. In dit proefschrift is geprobeerd te achterhalen hoe de PI-TP eiwitten betrokken zijn bij de productie van signaalmoleculen die effecten hebben op de regulatie van celgroei en celdood.

Toen PI-TPα tot overexpressie werd gebracht in muizen cellen gingen deze cellen veel sneller groeien en werden ze resistent tegen de inductie van apoptose. De muizen cellen met een overexpressie van PI-TPα worden SPIα cellen genoemd. Analyse van het medium, waarin de SPIα cellen 24 uur hebben gegroeid (geconditioneerd medium van SPIα cellen; CM van SPIα), wees uit dat deze cellen signaalmoleculen uitscheiden die groei stimuleren in andere cellen en die andere cellen ook resistent maken tegen de inductie van apoptose. De rol van PI-TPα in dit proces wordt gezien als het eiwit dat het lipide PI uit het membraan haalt en "aangeeft" aan een ander eiwit dat de één van de

vetten (arachidon-vetzuur-staart) van PI afknipt. De afgeknipte vetzuurstaart wordt vervolgens omgezet door cyclooxygenase naar een arachidonzuur-metaboliet en uitgescheiden in het medium waarin de cel groeit. Om vanuit dit medium een effect te kunnen hebben op een cel, moeten deze arachidonzuur metabolieten aan een receptor binden aan de buitenkant van de cel. Wanneer de SPIα cellen groeien in de aanwezigheid van een remmer, die er voor zorgt dat het eiwit cyclooxygenase niet meer werkt, wordt het medium van deze cellen minder groei-stimulerend en kan het minder goed bescherming bieden tegen de inductie van apoptose. De functie van cyclooxygenase is het omzetten van arachidonzuur (een van de vetzuurstaarten van PI) naar prostaglandines en andere arachidonzuur metabolieten. Het is gebleken dat de arachidonzuur metabolieten, waarvan de productie afhankelijk is van PI-TPα, binden aan de cannabinoid 1 receptor. Deze receptor, waaraan ook de actieve component van marihuana bindt, is betrokken bij de regulatie van de bescherming van cellen tegen celdood en is voornamelijk aanwezig in de hersenen.

In hoofdstuk 3 laten we zien dat de cannabinoid 1 receptor aanwezig is in de cellen die we gebruiken bij dit onderzoek (muis fibroblast cellen). De meeste cannabinoid receptoren werden gevonden in de cellen die de hoogste resistentie tegen geïnduceerde apoptose vertonen, de SPIα cellen. Na het activeren van de cannabinoid receptor is een cel nog niet resistent tegen apoptose. Het signaal moet eerst nog worden doorgeven naar de kern, waar dit signaal wordt vertaald naar de expressie/aanmaak van eiwitten die helpen om een cel te beschermen tegen apoptose. Het doorgeven van het signaal gebeurt door de activering van een reeks van opeenvolgende eiwitten, ook wel een signaal transductie pad genoemd. Wanneer cellen geïncubeerd/blootgesteld worden met/aan de signaalmoleculen die worden uitgescheiden door SPIα cellen, worden er minstens twee signaal transductie paden geactiveerd, het ‘p42/p44 MAP kinase pathway’ en het ‘Akt/PKB pathway’. In de literatuur worden deze paden beschreven als paden die worden geactiveerd, wanneer een cel beschermd wordt tegen apoptose. Deze paden geven het signaal, afkomstig van de cannabinoid receptor, door naar de kern. In de kern worden eiwitten (transcriptie factoren) geactiveerd die het signaal vertalen naar de expressie van eiwitten die een cel beschermen tegen apoptose. Eén van de eiwitten verantwoordelijk voor de “vertaling” van het signaal is de transcriptie factor NF-κB. In cellen die

geïncubeerd zijn met de signaalmoleculen die uitgescheiden worden door SPIα cellen wordt deze transcriptie factor geactiveerd.

Uit literatuur is bekend dat zowel PI-TPα als PI-TPβ gefosforyleerd kunnen worden (dit betekent dat een fosfaatgroep aan PI-TPα en PI-TPβ gekoppeld kan worden) door een enzym 'proteïne kinase C'. Het ontbreken van de fosfaatgroep op serine-166 (PI-TPα) en serine-165 (PI-TPβ) zorgt ervoor dat deze PI-TPs geen lipiden meer kunnen transporteren *in vitro*. Naast serine-165 heeft PI-TPβ nog een fosforylatie plek op serine-262, die niet aanwezig is in PI-TPα. Het ontbreken van een fosfaatgroep op serine-262 zorgt er voor dat PI-TP nog wel lipiden kan transporteren maar niet meer lokaliseert naar het Golgi apparaat. In contrast met de snel groeiende SPIα cellen, gaan muizen fibroblast cellen met een overexpressie van PI-TPβ (SPIβ cellen) juist langzamer groeien en worden erg gevoelig voor de inductie van apoptose. Een cellijn met een overexpressie van een mutant PI-TPβ waarin de serine-262 fosforylatie plek verwijderd is, SPIβS262A, heeft de groei eigenschappen en apoptose gevoeligheid die overeen komen met de wild-type (onveranderde) cellijn. Dit wijst erop dat de fosforylatie van PI-TPβ op serine-262 essentieel is voor de rol van PI-TPβ in de regulatie van celgroei en apoptose gevoeligheid. Een aantal aanwijzingen waarom SPIβ cellen zo gevoelig zijn voor de inductie van apoptose wordt beschreven in hoofdstuk 4. Behalve het geconditioneerde medium (CM) van SPIα cellen kan het CM van wild-type cellen ook bescherming bieden tegen apoptose in de SPIβ cellen, maar in mindere mate dan het CM van SPIα cellen. Hoewel de SPIβ cellen ook PI-TPα bevatten, heeft het CM van SPIβ cellen geheel tegen de verwachting in geen beschermende activiteit. Echter, nadat de eiwitten in het CM van SPIβ cellen gedenatureerd/geïnactiveerd waren door verwarming, bleek dit geconditioneerde medium toch beschermende activiteit te bezitten, vergelijkbaar met het CM van wild-type cellen. Dit betekent dat er in het CM van SPIβ cellen een 'antagonist' aanwezig is, die de beschermende activiteit aanwezig in het medium van SPIβ cellen tegenwerkt of maskeert. Aangezien het CM van SPIβS262A cellen (overexpressie van PI-TPβ zonder de fosforylatie plek op serine-262) deze 'antagonist' niet bevat, suggereert dat de productie en secretie van deze 'antagonist' afhankelijk is van de aanwezigheid van gefosforyleerd PI-TPβ.

Van twee arachidonzuur (vetzuur staart van lipide) metabolieten, prostaglandine E_2 en prostaglandine $F_{2\alpha,}$ waarvan we hebben aangetoond dat ze aanwezig zijn in het CM van SPIα, is bekend dat ze sommige cellijnen kunnen beschermen tegen apoptose. Zoals verwacht waren beide prostaglandines in staat de wilde-type cellen en de SPIβS262A cellen te beschermen en werd het anti-apoptotische 'p42/p44 MAP kinase' pad geactiveerd. Aan de andere kant waren deze prostaglandines niet in staat de SPIβ cellen te beschermen tegen apoptose en werd het 'p42/p44 MAP kinase' pad ook niet geactiveerd in deze cellen. Dit duidt erop dat specifieke mechanismen, verantwoordelijk voor de bescherming van cellen tegen apoptose, in de SPIβ cellen niet functioneren. Samenvattend kan worden gesteld dat de gevoeligheid van SPIβ cellen voor de inductie van apoptose wordt geassocieerd met de secretie van een 'antagonist' in het medium en het niet functioneren van anti-apoptotische paden.

De verschillende isolatie en identificatie methoden om de identiteit van de groei-stimulerende en beschermende moleculen te achterhalen, strandden vaak doordat de beschermende moleculen erg instabiel zijn wanneer ze uit hun natuurlijke milieu worden gehaald. Hoewel de identiteit niet is achterhaald, hebben alle verschillende methoden van zuivering wel veel informatie opgeleverd over chemische eigenschappen van deze moleculen. Zo is gebleken dat er meer dan één signaalmolecuul aanwezig is, dat beschermt tegen apoptose. Het moet nog blijken of deze verschillende moleculen alleen een cumulatief effect hebben of dat er een synergistisch effect optreedt. Tevens zijn een aantal mogelijke kandidaten geëvalueerd (2-arachidonyl glycerol, anandamide, prostaglandine E_2 en prostaglandine $F_{2\alpha}$), maar deze bleken niet de beschermende moleculen te zijn waar naar we op zoek waren.

Samenvattend kan worden vastgesteld dat het in dit proefschrift beschreven onderzoek meer duidelijk heeft gemaakt over de rol van PI-TPα en PI-TPβ in de regulatie van celgroei en apoptose. Het laat zien dat het eiwit PI-TPβ cellen gevoeliger maakt voor apoptose. Het feit dat apoptose nodig is voor de ontwikkeling van een embryo zou kunnen verklaren waarom muizen embryo's zonder PI-TPβ ver voor de geboorte sterven. Aan de andere kant laat die proefschrift zien dat PI-TPα cellen

ongevoelig maakt voor apoptose en dat PI-TPα verantwoordelijk is voor de productie en secretie van signaalmoleculen die andere cellen kunnen beschermen tegen apoptose. Het feit dat muizen embryo's zonder PI-TPα wel gezond geboren worden, kan mogelijk worden verklaard door het feit dat deze embryo's de beschermende moleculen van hun moeder ontvangen. Na de geboorte sterven deze muizen binnen 14 dagen aangezien ze, bij gebrek aan PI-TPα, de beschermende moleculen niet zelf kunnen maken waardoor hun neurale cellen afsterven.

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Martijn

Curriculum Vitae

Martijn Schenning werd geboren op 19 januari 1979, te Apeldoorn. Na het behalen van het gymnasium diploma in 1997 aan het Christelijk Lyceum te Apeldoorn, is hij scheikunde gaan studeren aan de Universiteit Utrecht. In 1998 werd het propedeutisch examen afgelegd en in 2002 werd het doctoraal diploma Scheikunde behaald. Vanaf augustus 2002 was hij werkzaam als Assistent-in-Opleiding bij de vakgroep Biochemie van Lipiden van het Bijvoet centrum, onderdeel van het instituut voor Biomembranen (IB), in dienst van de Universiteit Utrecht. Onder begeleiding van dr. G.T. Snoek en prof. K.W.A. Wirtz werd het in dit proefschrift beschreven onderzoek uitgevoerd. Tijdens deze aanstelling werd er twee maanden gewerkt aan de University of California, te Irvine (UCI) onder begeleiding van Giuseppe Astarita en prof. dr. D. Piomelli.

List of publications

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M. Schenning, C.M. van Tiel, K.W.A. Wirtz, G.T. Snoek. The anti-apoptotic MAP kinase pathway is inhibited in NIH3T3 fibroblasts with increased expression of PI-TPβ. *Submitted.*