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STATIN-INDUCED CELLULAR EFFECTS MEDIATED BY THE P2X7 RECEPTOR

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Cover: Confocal image of cells using Proximity Ligase Assay. Courtesy to Olink AB.

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To My Parents

ABSTRACT

Cholesterol-lowering statins have been shown to inhibit growth of different cancer cells both *in vitro* and *in vivo*. Epidemiological studies also indicate a chemopreventive effect of statins. We have investigated the effect of statins on Akt/protein kinase B signaling and their sensitizing effect of cytostatic drugs.

It was found that insulin- and cytostatic drug-induced Akt phosphorylations and nuclear translocation was inhibited by pravastatin and atorvastatin in HepG2, A549 and H1299 cells in an mTOR dependent manner. Statins also induced mTOR dependent phosphorylations of insulin receptor substrate 1 (IRS-1). In p53 wild-type cells (HepG2 and A549) pretreatment with statins did not sensitized cells to etoposide in concentrations which induced p53 stabilization. In line with our previous data, statins were found to attenuate the etoposide-induced p53 response. We also show that in a p53-deficient cell line (H1299) pretreatment with atorvastatin sensitized cells to etoposide, doxorubicin and 5-fluorouracil and increased the level of apoptosis. Taken together we clearly demonstrate, that an mTOR–dependent, statin-induced inhibition of Akt phosphorylation and nuclear localization sensitize cells to cytostatic drugs. However, this effect can be counteracted in p53 competent cells by the ability of statins to destabilize p53.

Further we address the question of possible anti-cancer effects of statins. We tested the possibility that statin-induced effects on nuclear pAkt are mediated by the P2X7 receptor. We show that low concentration of atorvastatin decreased the level of insulin-induced phosphorylated Akt in the nucleus. This effect was seen within minutes and was inhibited by P2X7 inhibitors. Our results reveal that statins via the P2X7 receptor modulate insulin-induced Akt signaling in epithelial cells. Furthermore, our data indicate that P2X7 regulate nuclear pAkt in epithelial cells.

We also invastigated the effect of statins in pancreatic cancer cell lines. We found that atorvastatin decreased constitutive- and insulin-induced pAkt in Panc-1 and MIA PaCa-2 cells. Statins also inhibited pAkt in synergy with gemcitabine- and 5 fluorouracil, and sensitized cells to apoptosis and inhibited cell proliferation. It was also found that the P2X7-purinergic receptor mediated the effects of statins in Panc-1 and MIA PaCa-2 cells. Interestingly, in Capan-2 cells, which expressed P2X7 in low levels, statins did not reduce pAkt levels nor did statins sensitize them to cytostatic drugs. However, statin inhibited the growth of Capan-2 cells and this correlated to inhibition of NFkB and Raf/MEK pathways. These effect might be explained by an inhibited protein prenylation.

Finally, we investigated the mechanism underlying the rapid inhibition of nuclear Akt. A role of PTEN as a negative regulator was suggested. Simultaneous activation of PTEN, PHLPP1 and 2, and calcineurin was detected in the time frame where pAkt is inhibited and abolished from nucleus. A model for how PTEN and PHLPP control Akt signaling has been suggests: PTEN prevents activation by removing the second messenger, PIP3, that activates Akt, and PHLPP inactivates Akt by direct dephosphorylation. Our data indicate, for the first time, that PTEN and PHLPP were activated simultaneously and bound the same substrate. Apparently both phosphatases participated in the down-regulation of nuclear pAkt in a rapid and coordinated action.

Overall, these studies yield a novel mechanism by which Akt acts upon statin stimulation.

LIST OF PUBLICATIONS

I. Roudier E, **Mistafa O,** Stenius U.
Statins induce mammalian target of rapamycin (mTOR)-mediated inhibition of Akt signaling and sensitize p53-deficient cells to cytostatic drugs.
Molecular Cancer Therapeutics, 2006;5:2706–15

II. **Mistafa O**, Högberg Johan, Stenius U.
Statins and ATP regulate nuclear pAkt via the P2X7 purinergic receptor in epithelial cells.
Biochemical and Biophysical Research Communications, 2008;365:131-6

III. **Mistafa O**, Stenius U.
Statins inhibit Akt/PKB signaling via P2X7 receptor in pancreatic cancer cells.
[Submitted]

IV. **Mistafa O**, Ghalali A, Kadekar S, Högberg J, Stenius U.
Statin-induced depletion of pAkt correlates to changes on protein phosphatases PTEN, PHLPP and Calcineurin.
[Manuscript]

CONTENTS

1. INTRODUCTION **1**

1.1 CANCER 1

1.2 STATINS 1

1.2.1 The mevalonate pathway 1

1.2.2 Statins and Cancer 2

1.2.3 Anticancer effect of statins 3

1.2.3.1 *In vitro* studies 3

1.2.3.2 *In vivo* studies 3

1.2.3.3 Clinical studies 3

1.2.4 Statins and Akt 4

1.3 AKT SIGNALING PATHWAY 4

1.3.1 Akt activation 5

1.3.2 Regulators of Akt 6

1.3.2.1 PTEN 6

1.3.2.2 Other regulators of Akt 7

1.3.3 Substrates of Akt 8

1.3.3.1 Akt and mTOR signaling 8

1.3.3.2 IRS-1 9

1.3.3.3 p53/Mdm2-loop 10

1.3.4 Akt and Cancer 11

1.4 P2 RECEPTORS 11

1.4.1 P2X Receptors 11

1.4.2 P2X7 Receptor Expression 12

1.4.3 P2X7 Receptor cellular response 12

1.4.4 P2X7 inhibitors 13

1.4.5 P2X7 receptor in cell growth and tumor models 13

2. AIM OF THE STUDY **14**

3. MATERIAL AND METHODS **15**

3.1 CELL LINE CULTURES (Paper I-IV) 15

3.2 PROTEIN ANALYSIS (Paper I-IV) 15

3.2.1 Western blotting Analysis 15

3.2.2 Immunoprecipitation-IP 15

3.3 VIABILITY ASSAYS (Paper I and III) 15

3.4 APOPTOSIS DETECTION- Morphological analysis 16

3.5 MICROSCOPY (Paper I-IV) 16
3.5.1 Immunohistochemical staining 16
3.5.2 Immunocytochemical staining 16

3.6 TRANSFECTION METHODS – siRNA approach 16

4. RESULTS AND DISCUSSION 18

4.1 Paper I: Statins induce mammalian target of rapamycin (mTOR)-mediated inhibition of Akt signaling and sensitize p53-deficient cells to cytostatic drugs 18

4.2 Paper II: Statins and ATP regulate nuclear pAkt via the P2X7 purinergic receptor in epithelial cells 20

4.3 Paper III: Statins inhibit Akt/PKB signaling via P2X7 receptor in pancreatic cancer cells 22

4.4 Paper IV: Statin-induced depletion of pAkt correlates to changes on protein phosphatases PTEN, PHLPP and Calcineurin 24

5. CONCLUSION 26

6. SIGNIFICANCE 28

7. FUTURE PERSPECTIVES 29

8. ACKNOWLEDGEMENTS 30

9. REFERENCES 32

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BzATP	3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate
CLL	Ehronic lymphocytic leukaemia
CREB	cAMP- responsive element binding protein
eIF4E	Human eukaryotic translation initiation factor 4E
eNOS	Endothelial nitric-oxide synthase
ERK	Extracellular signal-regulated kinases
FOXO	Forkhead transcription factor
GSK 3	Glycogen synthase kinase 3
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
IGF	Insulin Growth Factor
IGF-1	Insulin-like growth factor-1
IL1-β	Interleukin-1ß
IRS-1	Insulin Receptor Substrate - 1
KN-62	1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
Mdm2	Mouse Double Minute 2
MEK	Mitogen-activated protein kinase
mTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
NEDD4-1	Neural precursor expressed developmentally down-regulated 4-1
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa B
NLS	Nuclear localization signal or sequence
NSCLC	Non Small Cell Lung Cancer
oATP	Periodate oxidized- ATP
pAkt	Phosphorylated Akt
P2	Purinergic Receptor
PDGF	Platelet-derived growth factor
PDK-1	Phosphoinositide Dependent Protein Kinase 1
PHLPP	Peckstrin homology domain leucine-rich repeat protein phosphatase
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase
PTEN	Phosphatase and Tensin homolog deleted on chromosome Ten
RTK	Receptor Tyrosine Kinase
siRNA	Small interference RNA
VEGF	Vascular Endothelial Growth Factor

1. INTRODUCTION

1.1 CANCER

Cancer is the second leading cause of death in the world and an abundance of evidence suggests that lifestyle factors including smoking, obesity, refined-sugar diet and physical inactivity account for the majority of cancer cases. During cancer development, tumor cells acquire a number of phenotypic characteristics that allow them to proliferate both rapidly and limitlessly, invade the surrounding tissue, survive without their normal microenvironment, and finally, metastasize to secondary sites (Hanahan D and Weinberg RA 2000). These features are usually acquired progressively over an extensive period of time as a result of increasing genomic instability that leads to up-regulation of oncogenes and down-regulation of tumor suppressor genes. For the past years, different major signaling pathways that are altered during tumourigenesis have been elucidated, pathways that link to dysregulated processes such as proliferation and survival. Disruption of one of these pathways, protein kinase B (PKB)/Akt signaling, has now been documented as a frequent occurrence in several human cancers and the enzyme appears to play an important role in their progression.

1.2 STATINS

Cholesterol is essential for proper mammalian membrane structure, membrane function, hormone production, lipid digestion, and lipid transport, and it originates from diet and *de novo* synthesis. Biosynthesis typically contributes with two-thirds of the total cholesterol amount in humans (Simons K et al 2000). HMG-CoA reductase inhibitors, commonly referred to as the statins, are a class of drugs that inhibits the rate-limiting step of the mevalonate pathway essential for the synthesis of various compounds, including cholesterol (Goldstein JL et al 1990). Currently more than 25 million people worldwide are treated with statins to prevent cardiovascular disease.

1.2.1 The mevalonate pathway.

High blood cholesterol is one of the major risk factors for heart disease and a common drug to reduce elevated total cholesterol and LDL (low-density lipoprotein) cholesterol levels are statins. Statins decrease hepatic cholesterol production, which in turn leads to increased LDL receptor turnover, enhanced hepatic LDL-cholesterol uptake, and ultimately decreased plasma LDL-cholesterol level (Ness GC et al 1996).

Statins available by prescription include atorvastatin (Lipitor^{TM}), lovastatin (Mevacor^{TM}), pravastatin (Pravachol^{TM}), and simvastatin (Zocor^{TM}). Simvastatin accounts for about 75% of all statin users in Sweden (Apotekets rapport).

The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate, which is catalyzed by HMG-CoA reductase. As shown in figure 1, the mevalonate pathway produces various end products that are important for many different cellular functions. For some proteins to become active, they must first undergo prenylation (e.g., farynesylation or geranylgeranylation) to associate with the plasma membrane. This association is achieved by the addition of a farnesyl moiety (e.g. Ras) or a geranylgeranyl moiety (e.g. Rho) to the COOH terminus of the proteins. Blockade

of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors results in decreased levels of mevalonate and its downstream products and, thus, may have significant influences on many critical cellular functions.

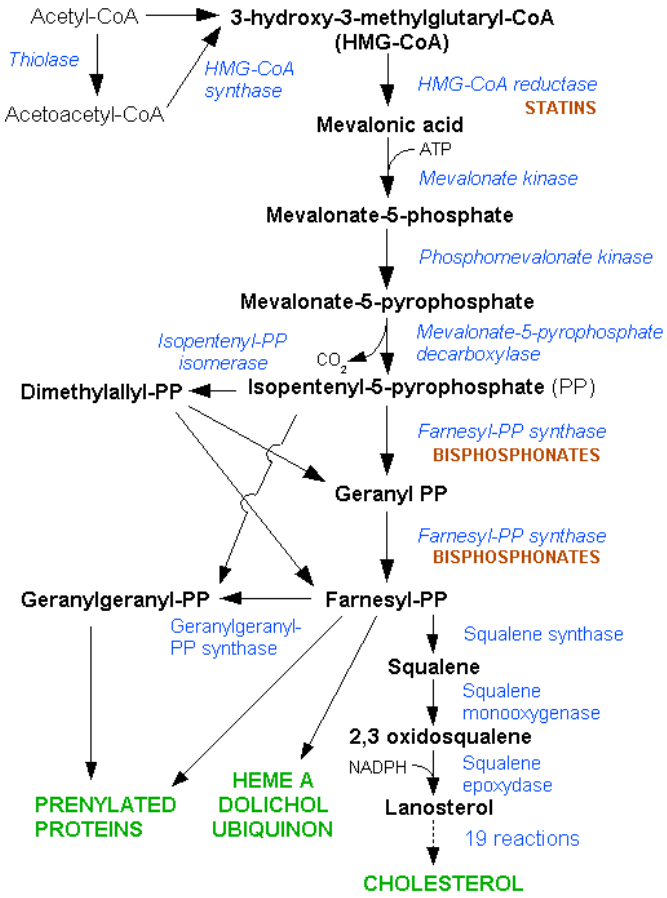

Figure 1: The mevalonate pathway (Berg JM et al 2002).

In recent years it has been showed that statins exert various effects on multiple targets, which are independent of their plasma cholesterol lowering properties (Ongini E et al 2004). A number of studies have shown that statins exert vascular protection through mechanisms involving NO production (Wolfrum S et al 2003). Some pleiotropic effects of statins appear to depend on the inhibition of the synthesis of isoprenoid intermediates of the mevalonate pathway that are critical for the posttranslational modification of several proteins, including the small GTP-binding Ras or Rho (Laufs U et al 1998; Nubel T et al 2004).

Besides that and based on experimental data and several clinical observations, it has been claimed that extra hepatic and cholesterol-lowering independent effects of statins might play a potentially beneficial role in cancer therapy (Graaf MR et al 2004; Jakobisiak M and Golab J 2003; Wong WW et al 2002).

1.2.2 Statins and Cancer

Known effects of statins on cardiovascular disease, along with years of strong evidence that these agents are relatively safe, have led researchers to explore whether statins

have the potential to prevent cancer. Indeed, statins have been shown to induce anti-proliferative, proapoptotic, anti-invasive and radiosensitising effects (Thibault A et al 1996). They also act as antioxidative, anti-inflammatory and angiogenic drugs and could therefore both prevent and promote cancer cell growth. There has been a growing interest in statins because of their possible anticancer effects (Chan KK et al 2003).

1.2.3 Anticancer effect of statins

1.2.3.1 *In vitro* studies

Low concentrations of statins have been shown to sensitize cancer cell lines to cytostatic drugs, such as 5-fluorouracil, taxol, etoposide, doxorubicin, and cisplatin (Holstein SA et al 2001; Feleszko W et al 2002; Khanzada UK et al 2006; Agarwal B et al 2002; Kozar K et al 2004; Wang W et al 2002). The interaction of statins with chemotherapeutic agents potentially enables the use of cytostatics in lower doses in the treatment of cancers that previously were only susceptible to apoptosis at unacceptably high doses.

1.2.3.2 *In vivo* studies

Besides their *in vitro* efficacy, statins have also been shown to have *in vivo* antitumor effects in different animal models. Efficacy in chemoprevention has been demonstrated in radiation-induced mammary tumorigenesis and chemical-induced colon tumorigenesis in rodent models (Inano H et al 1997; Narisawa T et al 1996). Statins have also been shown to have significant anti-proliferative effect on pancreatic tumor growth *in vivo* or syngeneic murine lung tumors (Issat T et al 2007, Bocci et al 2005; Kusama T et al 2002, Gbelcová H et al 2008; Hawk MA et al 1996). Statins have been shown to potentiate the antitumor effect of doxorubicin in three tumor models *in vivo* (Chan KK et al 2003). Inhibition of metastasis was also demonstrated on rat lymphoma, rat fibrosarcoma, mouse mammary tumor, hepatic metastasis of murine colon tumor, and mouse melanoma (Matar P et al 1999; Matar P et al 1998; Alonso DF et al 1998; Broitman SA et al 1996; Jani JP et al 1993). In addition, statins were shown to potentiate the antitumor effect of doxorubicin in three tumor models *in vivo* (Feleszko W et al 2002).

1.2.3.3 Clinical studies

In vitro and animal research as well as ongoing observations of people who take statins suggests that these drugs may lower the risk of certain cancers. Recent studies indicate a dramatic (< 50 %) risk reduction in metastatic or fatal prostate cancer and 80% reduced risk of pancreatic cancer among statin users (for more than 4 years), (Platz EA et al 2006; Khurana V et al 2007). Furthermore, in a case-control study, involving about half a million veterans it was found that > 6 months on statins reduced the risk of lung cancer by 55% (Khurana V et al 2007). In addition, simvastatin inhibited the small cell lung cancer tumor growth *in vivo* (Khanzada UK et al 2006).

Because statins exhibit such diverse effects on various aspects of carcinogenesis *in vivo*, numerous clinical trials have shown significant clinical benefit. Recently, a preclinical data translated to Phase I/II clinical trials demonstrated a promising result when statins were used in combination with standard chemotherapies (Schmidmaier R et al 2007; Kornblau SM et al 2007). Moreover, median survival was doubled with the addition of statins to 5-fluorouracil in advanced hepatocellular carcinomas (Kawata S et al 2001).

Although the great amount of publication showing the advantages of using statin *in vivo* and *in vitro*, a recently published review and meta-analysis suggests that statins do not have short-term effects on cancer risk (Kuoppala J et al 2008). Moreover, some of the evidence on potentially protective or harmful effects of statin was found inconclusive. Meta-analysis is a quantitative result and should be interpreted with caution. The major weakness of the method is the heavy reliance on published studies, which may decrease the effect of one study.

Preclinical data of statin on animals, including mouse, rat, rabbit, and dog, revealed linear pharmacokinetics. The therapeutic dose for the treatment of hypercholesterolemia is 1 mg/kg/day, which yields serum levels of 0.1 μM (Pan HY et al 1990). When the cytostatic activity of lovastatin was compared among a variety of cell lines, including melanoma, adenocarcinoma, and neuroblastoma, the IC50s were in the range of 0.3– 2.0 μM (Prasanna P et al 1996). This would seem to make statins a promising drug to be administered as a cytostatic agent in conjunction with other cytotoxic agents or radiation.

1.2.4 Statins and Akt

Recent studies indicate that statins and other cholesterol lowering drugs may influence the Akt/PKB signaling pathway in cancer cells by inhibition of Ras prenylation (Graaf MR et al 2004; Peres C et al 2003). For example, in breast cancer cells over-expressing Akt cerivastatin decreased the level pAkt (Denoyelle C et al 2003). In this study the authors suggest that anti-cancer action could be related to the inhibition of RhoA, by preventing RhoA prenylation. Another recent study shows that simvastatin could inhibit activation of Akt in small cell lung cancer cells by inhibiting Ras signaling (Khanzada UK et al 2006). Both PI3K/PKB and MEK/Erk signaling pathways mediate the effects of the drug on tumor cells. Statins may thus represent a potentially new and safe class of inhibitors of Akt signaling.

1.3 AKT SIGNALING PATHWAY

The maintenance of normal cell function and tissue homeostasis is dependent on the precise regulation of multiple signaling pathways that regulate cellular determination such as proliferation, differentiation, cell growth arrest, or programmed cell death (apoptosis and autophagy). Cancer arises from clones of mutated cells that break out this balance and proliferate inappropriately without compensatory apoptosis. Uncontrolled cell growth occurs as a result of disturbed signal transduction that modulates or alters cellular behavior or function to keep the critical balance between the rate of cell-cycle progression (cell division) and cell growth (cell mass) on one hand, and programmed cell death (apoptosis and autophagy) on the other (Hunter T 2000; Reed JC 1999).

Phosphoinositide 3-kinase (PI3K) plays a crucial role in effecting alterations in a broad range of cellular functions in response to extracellular signals. A key downstream effector of PI3K is the serine/threonine protein kinase, PKB/Akt, which has emerged as a crucial regulator of widely divergent cellular processes including nutrient metabolism, cell growth, proliferation, cell cycle control, survival, differentiation, migration, and angiogenesis (Datta SR et al 1999). Deregulation of the PI3K/Akt pathway has been related to the development of diseases such as diabetes,

autoimmunity, and cancer (Sengupta PS et al 2000; Shahin MS et al 2000; Obata K et al 1998; Kanuma Y et al 1997). The link between activation of the PI3K/Akt pathway and cancer makes this pathway an attractive target for therapeutic strategies. Accumulated reports show that activation of receptor protein-tyrosine kinases (RTK) and cytokine receptors are major resources for activating PI3K/Akt pathway.

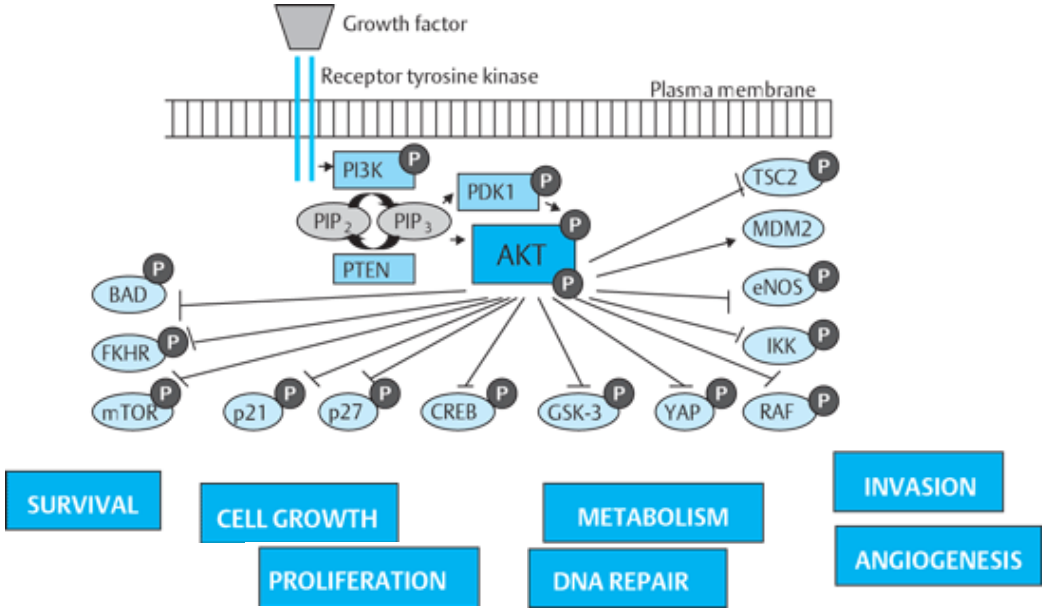

Figure 2: Schematic illustration of the Akt signaling pathway and pAkt targets (modified from Pitt SC and Chen H 2008).

1.3.1 Akt activation

Akt1 and Akt2, which were identified as human homologues of the viral oncogene *v-akt*, previously known to cause a form of leukemia in mice, were isolated from two genes (Staal SP 1987). Subsequently, three independent studies revealed that *v-akt* and its mammalian homologues encoded a protein kinase with some similarities to protein kinase C (PKC) and protein kinase A (PKA) and named it protein kinase B (PKB) (Bellacosa A et al 1991; Coffer PJ et al 1991; Jones PF et al 1991).

To date, three members of the family have been isolated and these are now referred to as PKBα (or Akt1), PKBβ (Akt2), and PKBγ (Akt3). The major structural features of the PKB/Akt proteins are illustrated in Fig. 2. Each isoform possesses an N-terminal pleckstrin homology (PH) domain of approximately 100 amino acids. Recent detailed structural examination of Akt PH domains reveals similarity to PH domains found in other signaling molecules that bind 3-phosphoinositides (Lietzke SE et al 2000; Ferguson KM et al 2000). This, together with evidence from earlier *in vitro* studies, indicates that the PH domain mediates binding of PKB/Akt to 3-phosphoinositides in the plasma membrane.

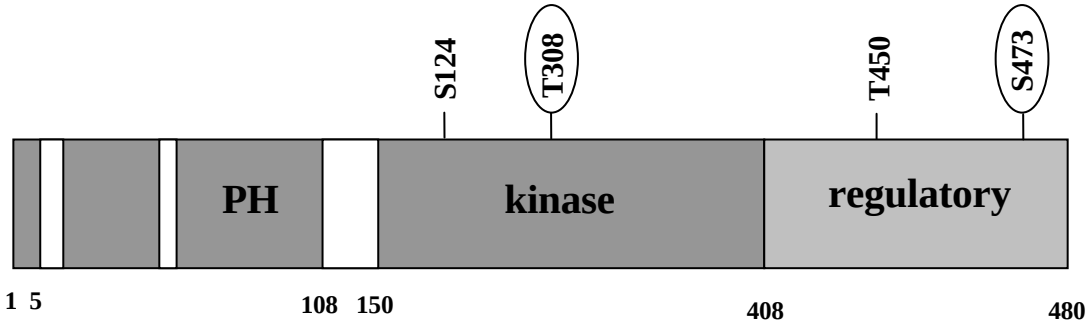

Figure 3: Domain structure of the human PKB/Akt isoform. Isoform consists of an N-terminal PH domain containing a region for binding inositol phospholipids, a kinase domain, and a C-terminal regulatory domain. Residues contained within a circle are serine and threonine sites inducibly phosphorylated in response to various stimuli.

The PH domain is followed by the kinase catalytic domain, which shows a high degree of similarity to those found in PKA and PKC (Jones PF et al 1991; Andjelkovic M et al 1995). Also present in this region is a threonine residue (Thr308) whose phosphorylation is necessary for activation of PKB/Akt. Following the kinase domain is a hydrophobic C-terminal tail containing a second regulatory phosphorylation site (Ser473), (figure 3). Phosphorylation of Akt (pAkt) at Thr308 and Ser473 occurs in response to growth factors (e.g. VEGF, PDGF, IGF) and other extracellular stimuli and is essential for maximal PKB/Akt activation (Alessi DR et al 1996). This can be prevented by PI3-kinase inhibitor, LY294002 (Downward J et al 1998; Nicholcon et al 2003; Forough R et al 2005; Xu, Q et al 2005). PKB/Akt may also be phosphorylated on Ser124 and Thr450 but neither of these sites appears to regulate PKB/Akt activity and their phosphorylation does not change following cell stimulation (Alessi DR et al 1996).

1.3.2 Regulators of Akt

1.3.2.1 PTEN

PTEN (Phosphatase and Tensin homolog) is a dual function lipid and protein phosphatase that was originally identified as a tumor suppressor gene frequently mutated in the advanced stages of a number of human cancers, particularly glioblastoma, endometrial, and prostate cancers. Moreover, germline mutations in PTEN induce the rare autosomal dominant inherited human cancer syndrome known as Cowden's disease, which is associated with increased risk of developing breast and other cancers (Simpson L et al 2001). PTEN classically converts phosphatidylinositol-3,4,5-trisphosphate (PIP3) in the cytoplasm to phosphatidylinositol-4,5-bisphosphate (PIP2), thereby directly antagonizing the activity of PI3 kinase (PI3K) (Leevers et al 1999) (see figure 2).The results of studies in which PTEN has been over-expressed in various cell lines suggest that PTEN acts as a tumor suppressor by inhibiting cell growth and increasing susceptibility to apoptosis (Weng LP et al 1999; Lu Y et al 1999).

The main physiological lipid substrate for PTEN is PIP3, the product of PI3K (Maehama T et al 1998). PTEN-null cells show elevated PIP3 levels and constitutive Akt activity, indicating that PTEN acts to restrain the pathway in unstimulated cells. Absence of PTEN also strongly correlates with activation of Akt in tumor cell lines (Lu Y et al 1999; Weng LP et al 1999). Conversely, reintroduction of PTEN in cells lacking PTEN down-regulates Akt phosphorylation as well as reverses the phosphorylation of Akt´s cellular substrates such as BAD (Vasko V et al 2004).

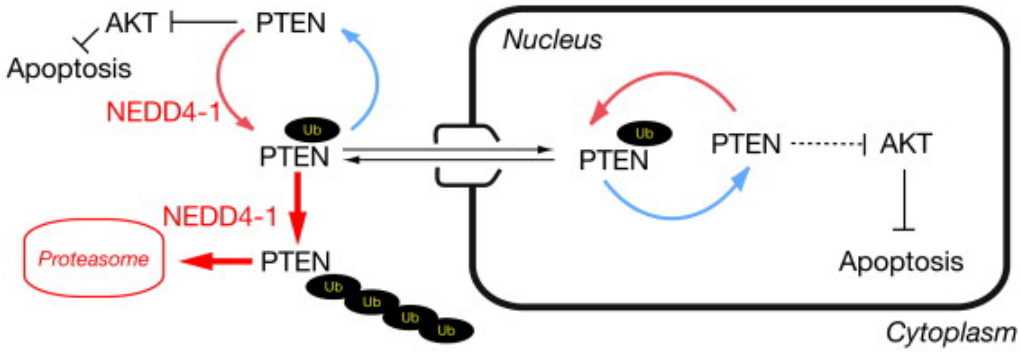

Figure 4: Model for regulation of PTEN import and shuttling versus PTEN degradation (Trotman LC et al 2007).

Several recent reports have brought conclusive evidence that PTEN, once believed to be a strictly cytoplasmic protein, shuttles to the nucleus and regulates PIP3 levels and hence Akt activity. PTEN does not contain nuclear import or export signals and several mechanisms have been suggested for nucleo-cytoplasmic shuttling of PTEN (Trotman LC, et al 2004; Chung JH et al 2005). Recently published data show that mono-ubiquitination mediated nuclear localization of PTEN is correlated with tumor suppression and inhibition of pAkt and that NEDD4-1 (neural precursor expressed developmentally down-regulated 4-1) regulates PTEN activity and localization (Corwell JA et al 2007; Wang X et al 20007; Ahn Y et al 2008), (see figure 4). Localization of PTEN may be regulated by other mechanisms, such as passive transport by diffusion (Liu F et al 2005) and active transport through NLS (nuclear localization signal), (Chung JH et al 2005). NEDD4-1 is a HECT domain protein and known as the first E3 ubiquitin ligase for PTEN. NEDD4-1 catalyzed mono-ubiquitination of PTEN which leads to its nuclear localization. This prevents from further poly-ubiquitination and degradation in the cytoplasm. NEDD4-1 may also poly-ubiquitinate PTEN in the cytosol which leads to a degradation of PTEN.

Finally, the termination of Akt signaling is as important as its activation. Many components of the PI3-kinase pathway such as the insulin receptor, insulin receptor substrates (IRS-1 and IRS-2), PI3-kinase, PTEN and PKB are capable of nuclear shuttling (Wu A et al 2003; Kim SJ 1998; Trotman LC, et al 2004; Meier R et al 1997; Podlecki D A et al 1987). Synthesis of PIP3 from PIP2 by nuclear PI3-kinase has been reported (Lu PJ et al 1998). These observations suggest that Akt nuclear localization may regulate nuclear events such as gene transcription.

1.3.2.2 Other regulators of Akt

The dephosphorulation reaction is catalyzed by the recently identified Ser473 PH domain and leucine-rich phosphatase (PHLPP) (Gao T et al 2005). The Ser/Thr-specific phosphatase PHLPP (pleckstrin homology domain leucine-rich repeat protein phosphatase) directly dephosphorylates the hydrophobic motif of Akt, resulting in inhibition of kinase activity and promotion of apoptosis. Interestingly, two PHLPP isoforms exist, PHLPP1 and PHLPP2. PHLPP1 and PHLPP2 have been shown to directly de-phosphorylate, and therefore acutely inactivate, distinct Akt isoforms, at one of the two critical phosphorylation sites required for activation: Ser473. PHLPP2 dephosphorylates Akt1 and Akt3, whereas PHLPP1 is specific for Akt2 and Akt3. It is

shown that both PHLPP isoforms are present in the cytosolic, nucleus and membrane fractions. A model of termination of Akt signaling has been presented. In this model PTEN dephosphorylates PIP3 and PHLPP dephosphorylates pAkt (Brognard J et al 2007; Brognard J et al 2008). Calcineurin is another phosphatase that might be involved in the regulation of Akt. A recent study suggests that Akt is a direct substrate of calcineurin in cardiomyocytes (Ni YG e al 2007).

1.3.3 Substrates of Akt

A couple of dozen proteins have been identified as Akt substrates, which include BAD, glycogen synthase kinase 3 (GSK3), caspase-9, endothelial nitric-oxide synthase (eNOS), IκB kinase, rac1, raf-1 protein kinase, mammalian target of rapamycin (mTOR), breast cancer susceptibility gene 1, insulin receptor substrate 1 (IRS-1), and forkhead transcription factors (FoxO), cAMP- responsive element binding protein (CREB) and Mdm2. Akt can potentially phosphorylate over 9000 proteins in mammalian cells (Martelli AM et al 2006), and it is not surprising that Akt activity can be detected in both the nucleus and cytoplasm. Although a number of physiological substrates of Akt have been reported, the detailed mechanism by which Akt regulates cell growth and survival has not been well documented, and putative critical substrates remain to be fully characterized.

1.3.3.1 Akt and mTOR signaling

PI3K/Akt has recently emerged as an important mediator to regulate nutrients- and growth factor-induced targeting of the TOR-pathway. TOR (mTOR in mammals, also known as FRAP) is a serine/threonine kinase that regulates both cell growth and cell cycle progression by integrating signals from nutrients (amino acids and energy) and growth factors (Heitman J et al 1991; Kunz J et al 1993; Helliwell SB et al 1994; Abraham RT 2002; Schmelzle T et al 2000).

mTOR is known to regulate protein translation by initiation of mRNA translation and ribosome synthesis leading to an increased rate of cell mass and size, which is required to support the rapid proliferation. It has been shown that mTOR regulates Akt. In *Drosophila* and mammalian cells, TOR and its associated protein rictor are necessary for Ser473 phosphorylation of Akt, and a reduction in rictor or mTOR expression inhibits Akt. The rictor-mTOR complex directly phosphorylated Akt/PKB on serine-473 *in vitro* and facilitated threonine-308 phosphorylation by PDK1 (Sarbassov DD et al 2005b).

mTOR and its downstream targets have recently been appreciated as an important cascade for tumorigenesis and novel therapeutic targets for cancer (Abraham RT 2002; Schmelzle T et al 2000). Using a murine lymphoma model, Akt promotes tumorigenesis and drug resistance by disrupting apoptosis and disruption of Akt signaling .Using the mTOR inhibitor rapamycin reverses chemo resistance in lymphomas expressing Akt, but not in those with other apoptotic defects (Wendel HG et al 2004). eIF4E (Human eukaryotic translation initiation factor 4E), a translational regulator that acts downstream of Akt and mTOR, recapitulated Akt's action in tumorigenesis and drug resistance but was unable to confer sensitivity to rapamycin and chemotherapy. Akt signals through mTOR and eIF4E as an important mechanism of oncogenesis and drug

resistance *in vivo* and reveals how targeting apoptotic programs can restore drug sensitivity in a genotype-dependent manner.

Since inhibition of mTOR might have direct effects on tumor cells manifest by slowing proliferation, increasing apoptosis or inhibiting tumor-derived vascular endothelial growth factor, a number of Phase I/II clinical trials with the rapalogs are ongoing (Easton JB and Houghton PJ, 2006).

1.3.3.2 IRS-1

Insulin receptor substrate-1(IRS-1) plays a key role in transmitting signals from the insulin and insulin-like growth factor-1 (IGF-1) receptors to intracellular PI3K / Akt and Erk/MAP kinase pathways (Sun XJ et al 1991; O'Connor R 2003).

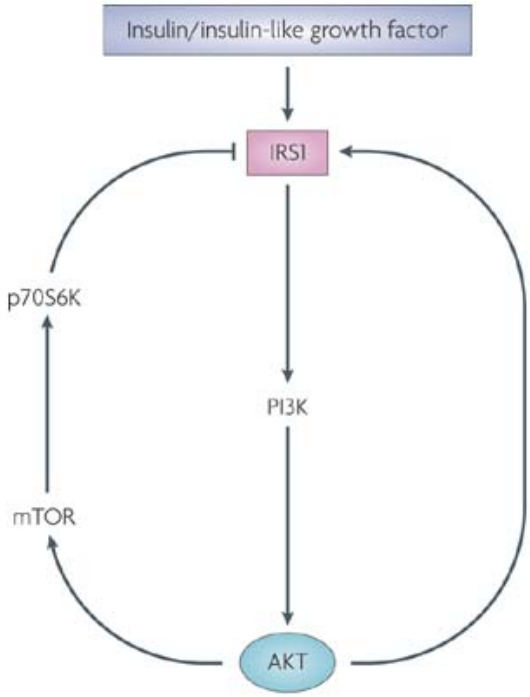

Figure 5: The scaffold protein insulin receptor substrate 1 (IRS-1) is regulated both positively and negatively by AKT by two distinct signaling axes, (Araujo RP et al 2007)

The activation of PKB in response to insulin propagates insulin signaling and promotes the phosphorylation of IRS-1 on a serine residue in turn generating a positive-feedback loop for insulin action (figure 5). Insulin also activates several kinases and these kinases act to induce the phosphorylation of IRS-1 on specific sites and inhibit its functions. This is part of the negative-feedback control mechanism induced by insulin that leads to termination of its action.

The activation of PI3K by IGF-1 is mediated by IRS-1 and IRS-2. IRS-1, especially when activated by the IGF-IR, sends an unambiguously mitogenic, anti-apoptotic and anti-differentiation signal (Baserga R 2000). IRS-1 expression is often increased in human tumors and a crucial role of IRS-1 in cancer has been proposed (Chang Q et al 2002). Over-expression of IRS-1 causes cell transformation, including the ability to form colonies in soft agar and tumors in mice (Dalmizrak O et al 2007). IRS-1 can act as a nuclear shuttling protein which can transport other proteins to the nucleus (Chen et al

2005). In a recent study it has been implicated that IRS-1 may bind Akt when translocated to the nucleus (Chen et al 2005).

1.3.3.3 p53/Mdm2-loop

The tumor suppressor p53 plays a key role in the DNA damage-induced apoptosis and cell cycle arrest in response to a variety of genotoxic stresses and to the activation of some oncogenes, such as Myc, thereby preventing the propagation of damaged cells (Sherr CJ 1998). p53 plays a central role in the response to genotoxic stress and may trigger apoptosis, a function that might be crucial for chemotherapeutic efficiency in p53 competent cells (Levine AJ et al 1997). The function of p53 is controlled by several mechanisms, including the regulation of p53 protein stability. Central to this process is Mdm2 (murine double minute), an ubiquitin ligase that targets p53 for ubiquitination and allows export of p53 from the nucleus to the cytoplasm, where p53 degradation by proteasomes takes place (Haupt Y et al 1997; Honda R et al 1997).

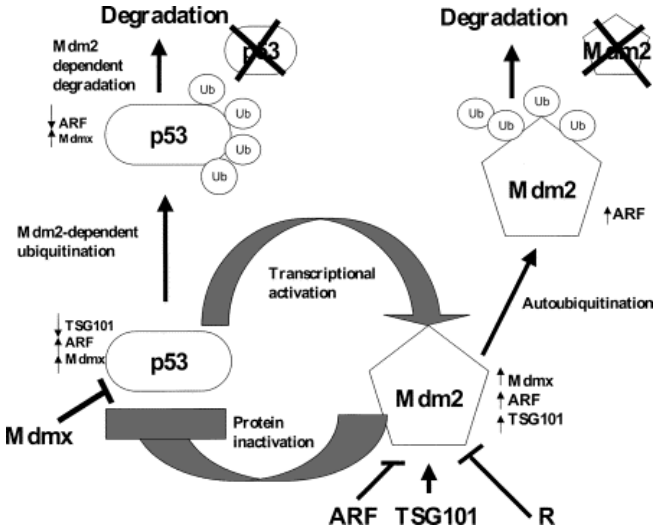

Figure 6: p53-MDM2 module and the ubiquitin system., (Michael D and Oren M 2003).

Under normal circumstances, p53 is maintained at very low levels by continuous ubiquitination and degradation. Activation of p53 in response to cellular stresses is mediated partly by inhibition of Mdm2 and rapid stabilization of p53 protein (figure 6), (Woods, D. B. et al 2001). p53 is one of the most important regulators for cell cycle progression and apoptosis in response to genotoxic stresses.

Results obtained previously by our group show that that statins alter Mdm2 expression and the p53 response to DNA damage. As a result, pravastatin induced Mdm2 phosphorylation at Ser166 in hepatocellular carcinoma (HepG2) cells (Pääjärvi G et al 2005). Mdm2 phosphorylation at Ser166 has been shown to activate Mdm2 and enhance its ubiquitination ligase function and destabilize p53 (Mayo LD et al 2001; Ogawara Y et al 2002; Zhou BP et al 2001). Moreover, Pääjärvi et al showed that statins attenuated the p53 response induced by genotoxicity *in vitro* and in rat liver *in vivo*. These effects were associated with an attenuated p21 response and lower level of apoptosis. Statins induce a phosphorylation of Mdm2 that may limit the duration and the intensity of the p53 response. Furthermore, this phosphorylation might contribute to nuclear localization of Mdm2 (Zhou BP et al 2001; Mayo LD et al 2001). Ogawara *et al.* show a PI3K/Akt-dependent increase of p53 ubiquitination (Ogawara Y et al 2002). Taken together, Mdm2 should

be, at least, an Akt target in the regulation of cell growth. Mayo LD et al 2001 showed that Akt activation leads to Mdm2 phosphorylation on Ser166, but the detailed mechanism needs to be further investigated

1.3.4 Akt and Cancer

As mentioned Akt is an anti-apoptotic factor activated in the plasma membrane by insulin, growth factors and cellular stress, and recent studies indicate a key role for Akt in e.g. carcinogenesis and as a target for therapeutic agents (Salmena L et al 2007; Lian Z et al 2005; Deleris P et al 2006; Corwell JAet al 2007; Trotman LC et al 2007). The Akt pathway is one of the major anti-apoptotic pathways in cells and Akt is constitutively active in many human tumors (Fresno Vara JA et al 2004; Vivanco I et al 2002; Vasko V et al 2004). Furthermore, many of the commonly used cytostatic drugs activate Akt in cancer cells (West KA et al 2002). Inhibition of Akt has been shown to sensitize cancer cell lines to chemotherapeutic agents, therefore Akt is an attractive target for cancer therapy (Harrington L et al 2005; Sarbassov DD et al 2005; Luo HR et al 2003; Krystal GW et al 2002, Warshamana-Greene GS et al 2005, Tsurutani J et al 2005; Luo J et al 2003; Castillo SS et al 2004).

1.4 P2 RECEPTORS

Nucleotides act as extracellular messengers, as they are released by many cell types and specifically stimulate a class of plasma membrane receptors, P2 receptors. This leads to diverse responses depending on cell type and receptor subtypes expressed (Abbracchio MP et al 1994; Muller CE 2000). As shown in figure 7, P2 receptors are activated by nucleotides, and on the basis of pharmacological, functional, and cloning data, two P2 receptor subfamilies have been described so far: P2Y and P2X (Ralevic V et al 1998).

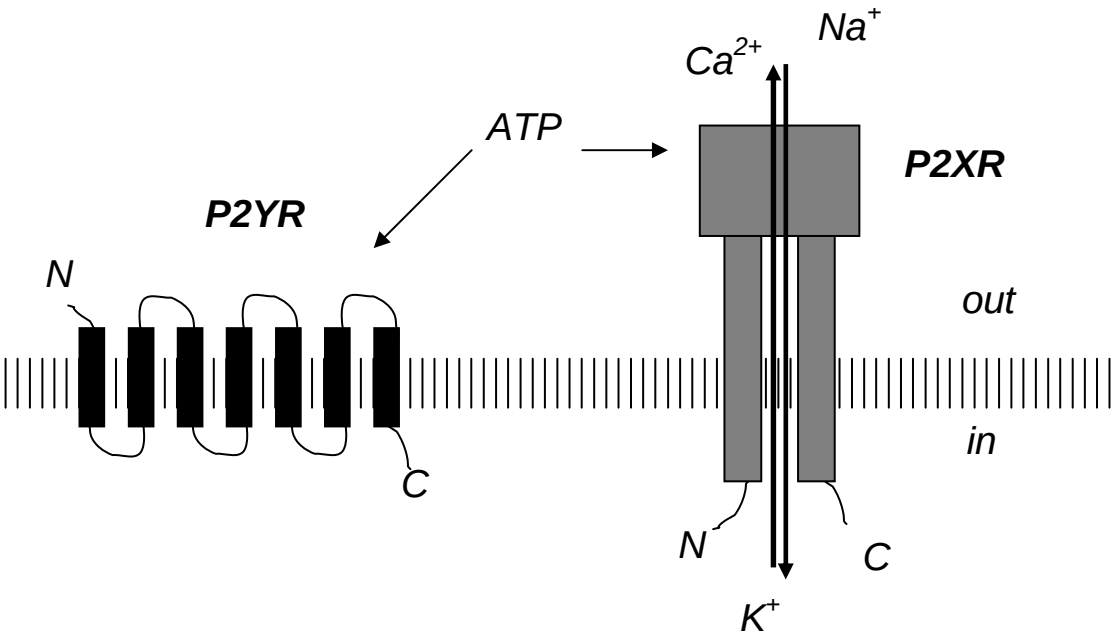

Figure 7: Schematic view of a G-Protein coupled P2Y receptor and an 'ionotropic' P2X receptor. The physiological ligand for both receptors is ATP and its metabolites.

1.4.1 P2X Receptors

P2X receptors are ligand-gated ion channels, selective for monovalent and divalent cations, and are activated by extracellular ATP and (Valera S et al 1994). Seven separate

genes coding for P2X subunits have been identified, and referred to as P2X1 through P2X7. All P2X subtypes are activated by ATP.

The purinergic P2X7 receptor belongs to the family of ATP-sensitive, ligand-gated ion channels and, like other members of the P2X family, mediates a non-selective cation conductance, when stimulated with an appropriate ligand (North RA 2002). The P2X7 receptor is present in a variety of cell types involved in pain, inflammatory processes and neurodegenerative conditions. Thus it may be an appealing target for pharmacological intervention.

The structure of P2X7 slightly differs from the other members of the P2X subfamily by its size (595 amino acids), and its long cytoplasmic carboxy-terminal tail (242 residues) (Surprenant A et al 1996). Brief exposure of P2X7 to agonists is responsible for a short opening of cation channels, whilst prolonged agonist exposure results in a sustained formation of a non-selective pore, allowing plasma membrane fluxes of ions as well as of hydrophilic molecules with molecular masses up to 900 Da.

1.4.2 P2X7 Receptor Expression

The P2X7 receptor were originally identified in mammalian sensory neurons, and later subsequently found expressed by a variety of cell types as different as neurons, macrophages, dendritic and microglial cells, smooth muscle cells, immune cells, fibroblasts, lymphocytes and endothelial cells (Di Virgilio F et al 2001; North RA 002; Adinolfi E et al 2005). At least a subpopulation of human osteoblasts also expresses P2X7 (Gartland A et al 2001). Expression of the receptor has been demonstrated in human fibroblasts and epithelia from the human bladder, human and rat uterus, male genital organs of the rats, human fetal keratinocytes and mouse parotid acinar and duct cells (Solini A et al 2000; O'Reilly BA et al 2001; Slater NM et al 2000; Tassell W et al 2000; Lee HY et al 2000; Greig AV et al 2003; Li Q et al 2003).

Increased levels of P2X7 were recently detected in chronic pancreatitis and in pancreas cancer, indicating a possible involvement of P2X7 receptors in pancreatic cancer development (Künzli et al 2007). In addition it also has been showed that rat hepatocytes and human prostate cells express functional P2X receptors (Gonzales E et al 2007).

1.4.3 P2X7 Receptor cellular response

P2X7 receptors are activated by ATP and BzATP. Depending on cell background, activation of the P2X7 receptor triggers diverse physiologic processes. For example, human monocytes primed by bacterial endotoxin/ lipopolysaccharide (LPS) respond to extracellular ATP with the caspase1 dependent proteolytic maturation and externalization of IL1-β. (Hogquist KA et al 1991; Perregaux D et al 1994; Ferrari D et al 1997). Apoptosis is another well-known consequence of P2X7 receptor activation in various types of leukocytes (Zanovello et al. 1990; Zheng et al. 1991).

Subsequent studies have specifically implicated the P2X7 receptor in mediating ATP-induced apoptosis of human macrophages, mesangial calls, dendritic cells and microglial cells (Lammas DA et al 1997; SchulzeLohoff E et al 1998; Coutinho-Silva R et al 1999).

P2X7 receptors agonists induce nuclear accumulation of various activated transcription factors, such as NFAT within minutes or NFκB within hours (Ferrari D et al 1997; 1999).

1.4.4 P2X7 inhibitors

There are few antagonists selective for subtypes of the P2X receptors. Pharmacological inhibitors of the receptor have different properties and show diverse species/specificity, i.e. periodate oxidized- ATP (oATP), 1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl- L-tyrosyl]-4-phenylpiperazine (KN-62), and brilliant blue G.

Oxidized ATP, inhibits not only at P2X7 but also other P2X subtypes. KN-62 only inhibits human P2X7, while brilliant blue G is active at the murine subtype (Murgia M et al 1993; Humphreys BD et al 1998; Jiang LH et al 2000). A further proof of the existence of the receptor was given by the demonstration that blockade of P2X7 by a specific monoclonal antibody fully abrogate ATP-mediated cytotoxicity. Furthermore, cells lacking reactivity to anti-P2X7antibodies were also resistant to the ATP permeabilizing and cytotoxic effects (Buell G et al 1998). P2X7 would be a cytotoxic receptor, capable of killing the cell by forming membrane pores.

1.4.5 P2X7 receptor in cell growth and tumor models

In apparent contrast with the role of P2X7 receptor in necrosis and apoptosis, an increasing number of reports have also correlated this protein with increased cell proliferation and tumor transformation. The first cellular model in which a role for P2X7 in cell growth was suggested was T lymphocytes (Baricordi OR et al 1996).

Patients affected by the aggressive form of chronic lymphocytic leukaemia (CLL) showed accordingly higher resting calcium levels and ATP evoked calcium influx as well as higher sensitivity to ATP-mediated cytotoxicity. The proposed model predicts that a tonic, low activation of P2X7 receptor will lead to an increased proliferation, while an acute stimulation with high concentrations of nucleotide causes death of tumor lymphocytes (Adinolfi E et al 2002).

Several other tumors show an altered expression of the P2X7 receptor. For example, non-melanoma skin cancers express P2X7 receptor and die upon massive application of ATP or BzATP (Greig AV et al 2003). In this case the receptor seems to be associated with cells undergoing apoptosis as it co-localizes with TUNEL and active caspase-3 staining (Greig AV et al 2003). For other widely diffused neoplasia, such as prostate and breast cancer, the expression of P2X7R receptor has been immunologically detected specifically in transformed cells (Slater NM et al 2000; Slater M et al 2004). One hundred fourteen out of 116 prostate cancer biopsies stained positively for the P2X7 receptor and also cells well distinct from the tumor show expression of the receptor along with tumor progression (Slater M et al 2004). Likewise, all cases of in situ lobular and ductal carcinoma showed intense P2X7 labeling in the nuclei and cytoplasm, while more aggressive forms tended to present the receptor at the cell surface (Slater NM et al 2000). The authors of these studies infer that the expression of the P2X7 receptor could be an attempt of cancer cells to undergo apoptosis that fails because the receptor might be nonfunctional.

2. AIM OF THE STUDY

Cholesterol lowering statins have been shown to have anticancer effects in different models and sensitize human tumor cells to cytostatic drugs.

The overall aim of this project is to characterize the effect of statins on cellular signaling.

In **paper I**, the aim was to investigate the effect of statins on Akt signaling and their sensitizing effect of cytostatic drugs. Inhibition of Akt has been shown to sensitize cancer cell lines to chemotherapeutic agents and statins have been shown to increase the efficiency of 5 fluorouracil, cisplatin, doxorubicin, etc. However, p53 stabilization may play a central role in the toxicity of these genotoxic drugs. Therefore, the influence of p53 on the statin-induced sensitizing effect was also studied.

The aim of **paper II** was to test the possibility that statin-induced effects on nuclear pAkt are mediated by the P2X7 receptor. P2X7 is activated by extracellular purinergic nucleotides such as ATP, and the release of ATP in the blood stream leads to pleiotropic cellular effects at least partially overlapping those induced by statins. The activation of P2X7 by statins may explain many pleiotropic effects.

In **paper III**, the aim was to investigate if the findings in paper I and II could be generalized to pancreatic cancer cell lines. Pancreatic cancer is associated with poor prognosis and does not respond to conventional chemotherapy. Increased levels of P2X7 were recently detected in chronic pancreatitis and pancreas cancer.

The aim of the last study, **paper IV**, was to study the mechanism of statin-induced pAkt deactivation.

3. MATERIAL AND METHODS

Detailed descriptions of all the techniques used in this thesis can be found in Papers I-IV presented. Here is a brief list and description with commentaries on the materials and methods.

3.1 CELL LINE CULTURES (Paper I-IV)

Different cell lines were used. In **papers I**, **II** and **III**, non-small cell lung cancer cells, A549 cells were used. In **papers I** and **II**, the following cell lines were also used; the p53 null H1299 human lung adenocarcinoma and hepatocellular carcinoma, HepG2 cells. In **paper III**, three different ductal cell lines of pancreatic origin were used: Panc-1, MIA PaCa-2 and Capan-2. In **paper II** and **III** human embryonic kidney (HEK) 293 cells stably expressing human P2X4 and P2X7 were used.

A549, H1299 and HepG2 cells were cultured as described by Roudier E et al 2006. Human embryonic kidney (HEK) 293 cells stably expressing human P2X4 and P2X7 were kindly provided by A. Surprenant, Sheffield University, UK. HEK293 cells were grown in DMEM: F12 with 1 mM l-glutamine, 10% inactivated calf serum and 300 μg/ml G418. Panc-1 and MIA PaCa-2 cells were maintained in Dulbecco's modified Eagle's medium (ATCC) while Capan-2 cells were maintained in McCoy's 5A Modified Medium (ATCC). Media was supplemented with 10% fetal calf serum and penicillin-streptomycine. Serum-starved cells were cultured in medium supplemented with 0.1% (A549, H1299, Panc-1) serum for 24 h or 0.5% serum for 48 h (HepG2). Through out the experiments, cells were grown in a humidified 5% CO_2-atmosphere at 37^{o}C.

3.2 PROTEIN ANALYSIS (Paper I-IV)

3.2.1 Western blotting Analysis

Western blotting is an analytical technique for detection of specific proteins in a given sample. In brief the samples were subjected to SDS–PAGE and thereafter blotted onto a PVDF membrane (Bio-Rad, Hercules, CA). Some samples were sub fractionated. The protein bands were probed using different antibodies. Proteins were visualized with ECL procedure (Amersham Biosciences, Uppsala, Sweden). The Western blotting results were analyzed with NIH Image 1.62 software.

3.2.2. Immunoprecipitation - IP

Immunoprecipitation was used in **paper IV**. In immunoprecipitation experiments, antibodies specific for a protein and beads coated in Protein A/G were added directly to the protein mixture to bind their targets overnight. The bead-antibody-protein complex were separated from the sample by centrifugation and analyzed by SDS-PAGE.

3.3 VIABILITY ASSAYS (Paper I and III)

Viability of cells was determined by the trypan blue exclusion assay. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay detecting the cellular mitochondrial capacity to convert MTT tetrazolium

salt to formazan. Cells were incubated with the medium containing MTT (Sigma-Aldrich, St. Louis, MO) for 4 hours. The cells were then lyzed in DMSO. The absorbance was measured at 570 nm.

3.4 APOPTOSIS DETECTION- Morphological analysis (Paper I and III)

Morphological evaluation of apoptotic cell death was performed by Hoechst staining. Cells were fixed in methanol for one hour. After fixation the cells were stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) for 5 minutes. Nuclear morphology was then evaluated using fluorescent microscope. Cells whose nucleus exhibited brightly stained, condensed chromatin or nuclear fragments were designated apoptotic. The percentage of cells with apoptotic morphology was determined by counting at least 1000 cells per plate.

3.5 MICROSCOPY (Paper I-IV)

3.5.1. Immunohistochemical staining

Female Sprague–Dawley rats were treated with pravastatin (4 mg/kg body weight) twice, 24 and 1 h before death. Fixed liver sections were incubated overnight with primary antibody. Primary antibodies were visualized using the EnVision+™ peroxidase kit (DAKO). The experimental protocol was approved by the Swedish Board of Laboratory Animals and was in accordance with National Institute of Health guidelines.

3.5.2 Immunocytochemical staining

Cells were fixed in 3.7% formaldehyde. After fixation the cells were stained with polyclonal antibodies against different antibodies. After incubation with primary antibodies, secondary antibody conjugated with FITC was applied (Dako, Glostrup, Denmark).

Double staining was performed on A549 cell. After fixation and after blocking the nonspecific binding, the sections were incubated with mouse and rabbit primary antibodies at 4°C overnight. Thereafter, the secondary antibodies, goat anti-rabbit (Alexa Fluor 488) or goat anti-mouse (Alexa Fluor 594), were applied. No staining was detected when the primary antibodies were omitted. The staining intensity was analyzed with NIH Image 1.62 software.

PLA was performed to image protein–protein interactions using microscopy. Proximity ligation was performed according to the manufacturer's protocol using the Duolink Detection Kit with PLA PLUS and MINUS Probes for mouse and rabbit (Olink Bioscience).

3.6 TRANSFECTION METHODS – siRNA approach

Small/short interfering RNA (**siRNA**) play a variety of roles in biology. siRNA is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene. The transfected siRNA efficiently knockdown the gene expression by binding to the key sequences on messenger RNA of proteins of interest.

This method was used in **paper I**, for efficient silencing of p53, in **paper II** and **II** for P2X7, and **paper IV** for PTEN and NEDD4-1 silencing. In all experiments, scrambled siRNA was used as a negative control and transfection was performed according to the TranIT-TKO protocol from the manufacturer (Mirus, Madison, WI). The level of protein expression in targeted cells was monitored by MTT and SDS-PAGE.

4 RESULTS AND DISCUSSION

4.1 Paper I: Statins induce mammalian target of rapamycin (mTOR)-mediated inhibition of Akt signaling and sensitize p53-deficient cells to cytostatic drugs.

In Paper I, we found that statins not only inhibits pAkt induced by insulin and cytostatic drugs, but its nuclear localization. This effect was documented employing antibodies specific for both Ser473 and Thr308 phosphorylated Akt. Akt phosphorylates substrates including regulators of apoptosis and growth in both cytoplasmic and nuclear compartments. It has been demonstrated that following activation through phosphorylation of Ser473 and Thr308, Akt detaches from the plasma membrane and translocates to the nucleus and that this translocation is crucial for its mitogenic activity (Meier R et al 1997). Several nuclear targets for Akt involved in the cell cycle regulation, such as p27 and FOXO have been identified (Vlahos CJ et el 1994). Exclusion from the nucleus may thus restrain the kinase activity on targets involved in regulation of the cell cycle.

Although statins have been shown to have anticarcinogenic effects in several models and many studies have shown their anti-proliferative and proapoptotic effects, the mechanisms for these anticancer effects are still not well characterized (Chan KK et al 2003, Demierre M-F et al 2005). In paper I, we investigated the effect of statins on the constitutive level pAkt in HepG2 and NSCLC cells (A549 and H1299). We also examined if pretreatment of statin could sensitize cells to cytostatic drugs.

Results in paper I clearly indicate that incubation of cells with low non-toxic concentration of pravastatin decreased a high constitutive level of pAkt on residue Ser473 (pAkt Ser473) and Akt on residue Thr308 (pAkt Thr308). To induce Akt phosphorylation, cells were incubated with insulin or cytostatic drugs. Preincubation of cells with statin for 1 hour decreased the level of insulin- and etoposide-induced pAkt Ser473. These observations indicate that pravastatin and atorvastatin attenuate the insulin and etoposide induced Akt kinase signaling in HepG2, A549 and H1299 cells.

The effect of statin on the anti-proliferative effects of etoposide, 5-Fu and doxorubicin were studied in HepG2 cells. All three cytostatic drugs induced a dose-dependent inhibition of cell proliferation. One hour pretreatment with pravastatin potentiated the etoposide-induced inhibition of cell proliferation. However, this effect was only detected in the lower concentration range. In previous publication published by our group it has been showed that statins can induce an mTOR-dependent attenuated p53-response to DNA damage (Pääjärvi G et al 2005). Therefore, the effect of pravastatin on etoposide-induced p53 stabilization was studied. Cytostatic induced a dose-dependent stabilization of both p53 and its downstream target p21. Clear p53 stabilization was induced by higher doses, while no significant p53 or p21 stabilization was observed at lower concentrations. Hence, the p53 response was not activated by cytostatics drugs in concentrations that were potentiated by pravastatin. This suggests that the ability of pravastatin to increase the anti-proliferative effect of cytostatic drugs could be dependent of the p53 status of the cells. To further elucidate the role of p53 status for

the effect of statins on cytostatic efficiency of drugs, p53-deficient H1299 cells were studied. In the H1299 cells, a significant increase of efficiency was observed for the whole range of etoposide concentrations. These observations indicate that statins potentiate the effect of commonly used cytostatic drugs in p53-deficient cells.

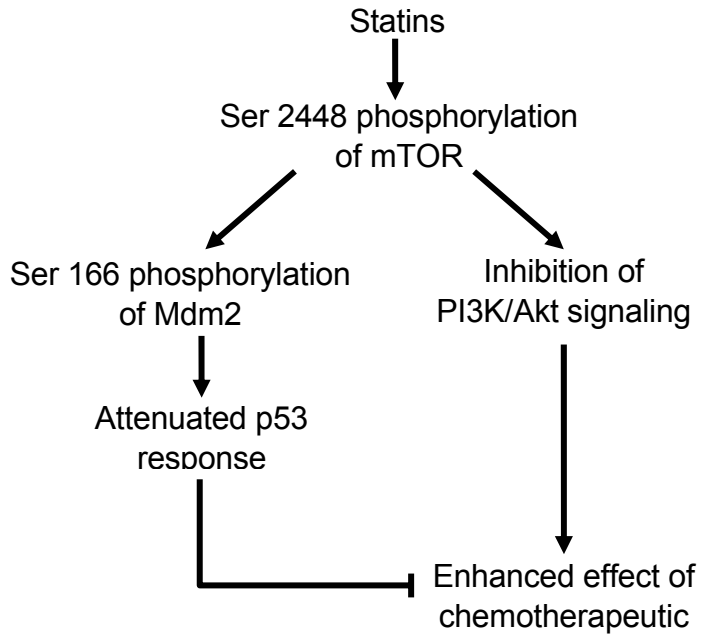

Figure 8: Statin-induced effects on p53 and Akt signaling.

Since nuclear translocation of Akt is crucial for its activity (Meier R et al 1997), we tested the possibility if statins can affect the nuclear location of Akt. We found that in insulin- or etoposide-treated cells a nuclear localization of pAkt Thr308 was induced. The number of pAkt-positive cells was decreased by 5 min pretreatment with atorvastatin and that effect lasted at least 45 minutes. Previous data from our group show that the phosphorylation of mTOR is induced by pravastatin in HepG2 cells (Pääjärvi G et al 2005). Hence, the role of mTOR in the inhibited nuclear translocation was studied. Here we found that an inhibitor of mTOR, rapamycin, and depletion of mTOR by siRNA, abrogated the atorvastatin-induced cytoplasmic localization of pAkt Thr308 in A549 cells and HepG2 cells. The effects of statins on mTOR, p53 and PI3K/Akt signaling are summarized in Figure 8.

IRS-1 phosphorylation has been shown to mediate mTOR-induced inhibition of Akt signaling (Tzatsos A et al 2006) and the treatment with rapamycin inhibited IRS-1 phosphorylation and abrogated the atorvastatin-induced inhibition of Akt. We also found that statin treatment induces IRS-1 phosphorylation at residue Ser 636/639 in cells. Statin and insulin treatment induced additative IRS-1 phosphorylation at residue Ser636/639 and this effect was inhibited by rapamycin.

Taken together, these findings suggest that pharmacologically relevant concentrations of statins can inhibit the activity of Akt induced by etoposide in hepatocellular carcinoma cells as well as in lung cancer cells in an mTOR dependent way.

4.2 Paper II: Statins and ATP regulate nuclear pAkt via the P2X7 purinergic receptor in epithelial cells.

The noticeable finding in Paper I was that atorvastatin affected nuclear levels of pAkt within 5 minutes and that the effect lasted at least 45 minutes. Most cardiovascular effects can be attributed to their HMG-CoA-reductase-inhibiting properties, leading to low levels of cholesterol and/or intermediary metabolites affecting cell signaling (Demierre M-F et al 2005, Platz EA et al 2006). However, HMG-CoA-reductase inhibition can hardly explain a number of rapid pleiotropic effects of statins. These effects are seen within seconds or minutes and include the binding of statins to the leukocyte LFA-1 receptor (Weitz-Schmidt G et al 2006), activation of eNOS (Harris MB et al 2004), and increased intracellular [Ca^{2+}] (Alvarez de Sotomayor M et al 2001).
Therefore in the second paper, we aimed to study the effect of statins in epithelial cells. We found that insulin-induced nuclear pAkt levels significantly decreased within one minute after addition of atorvastatin. Similar effects were induced by pravastatin and they were not prevented by mevalonate, a cholesterol precursor antagonizing HMG-CoA reductase inhibition (Paajarvi G et al 2005). Further we found that the level of nuclear pAkt Ser437 was decreased without a concomitant increase of pAkt in the cytoplasmic fraction. These experiments show that nuclear pAkt is a sensitive indicator of effects induced by statins in pharmacologically relevant concentrations.

To explain the rapid effect of statin we tested the possibility that the effect was mediated by P2X7 receptors. P2X7 purinergic receptors have been shown to induce rapid effects on cell signaling (Garcia-Marcos M et al 2006). The P2X antagonist, oxidized-ATP (o-ATP), and a more selective inhibitor of P2X7 receptor, KN-62, were tested (Slater M et al 2005). Results clearly demonstrate that o-ATP and KN-62 abrogates the atorvastatin-induced effect on pAkt Thr308 in insulin-treated A549 cells. Since ATP is a natural ligand for P2X receptor, ATP, and a more selective agonist for P2X7, BzATP, were tested. The insulin-induced nuclear pAkt Thr308 accumulation was completely abolished by agonists. These data indicate that extracellular ATP and BzATP induce similar effect as statins on nuclear pAkt. Since ATP may activate both P2Y and P2X receptors we tested an agonist of P2Y receptors, UTP. However, UTP did not have any effect on nuclear pAkt indicating a selective involvement of P2X and in particular P2X7 receptor.

A nuclear localization of P2X7 has been observed previously (Franke H et al 2004, Atkinson L et al 2002). Our *in vivo* data from rat hepatocytes clearly show a statin-induced nuclear localization of P2X7 and that statin affect the antigen. To further confirm an involvement of P2X7 receptors we used HEK293 cells heterologously expressing human P2X7 or P2X4 receptors (Jiang LH et al 2000). P2X4 and P2X7 receptor subunits are known to co-localize in immune, epithelial and exocrine gland cells (Jiang LH et al 2000). In HEK293 P2X7 cells atorvastatin decreased insulin-induced nuclear pAkt within 5 min. Atorvastatin did not decrease pAkt in cells expressing P2X4, implicating that the effect is mediated through P2X7 receptors.

These data corroborates the data given above and also provide evidence for an involvement of P2X7 receptor. Taken together these data indicate a major, if not exclusive, role for P2X7 in the effects shown above. In addition, we found that the

rapid inhibition of nuclear pAkt was not inhibited by mevalonate. The pleiotropic actions of statins may be both dependent and independent of the HMG-CoA reductase inhibition. Here we clearly show that the rapid effect of statins argue for a HMG-CoA reductase-independent effect of statins. Even though we found that statins affect P2X7 receptors it is not clear how P2X7 affect nuclear pAkt.

4.3 Paper III: Statins inhibit Akt/PKB signaling via P2X7 receptor in pancreatic cancer cells.

In a recent publication, increased levels of P2X7 were detected in chronic pancreatitis and pancreas cancer indicating a possible involvement of P2X7 receptors in pancreatic cancer development (Künzli et al 2007). Therefore, in **paper III**, we have investigated the effect of statins on Akt/protein kinase B signaling in pancreatic cancer cell lines.

PI3K/Akt pathway has been implicated in the resistance of pancreatic cancer to cytostatic drugs (Ng SSW et al 2000). We found that atorvastatin decreased constitutive- and insulin-induced pAkt in Panc-1 and MIA PaCa-2 cells as well as they downstream targets, pGSK3β Ser9 and p70 S6K Thr386. No effects by statin on neither pAkt nor downstream targets were detected in Capan-2 cells.

The effect of atorvastatin on cell proliferation was also studied. Incubation with low concentrations of atorvastatin for 48 hours inhibited cell proliferation dose-dependently in all three cell lines. We also show that statin induce a dose-dependent increase in the levels of PARP cleavage. As described in paper I and II, we found that statins affect the nuclear localization of pAkt. Together, these data show that atorvastatin attenuate Akt signaling in Panc-1 and MIA PaCa-2 cells, but not in Capan-2 cells

Statins also inhibited pAkt in synergy with gemcitabine- and 5 fluorouracil, and sensitized cells to gemcitabine- and 5 fluorouracil-induced apoptosis and inhibition of cell proliferation. Taken together, atorvastatin increased the effect 5-Fu or gemcitabine on cell proliferation and apoptosis in pancreatic cancer cells. This is in line with another publication showing that statins sensitize pancreatic cancer cell lines to cytostatic treatment (Chan KK et al 2003).Our data is also in line with a recently published study showing that fluvastatin inhibits proliferation, induces apoptosis and potentiates the cytotoxic effect of gemcitabine in MIA PaCa-2 cells (Bocci G et al 2005).

In line with data presented in Paper II, we found that the P2X7-purinergic receptor mediated the effects of statins in Panc-1 and MIA PaCa-2 cells. Thus, experiments employing siRNA approach and inhibitors supported an involvement of P2X7. We found that Capan-2 cells, in contrast to Panc-1 and MIA PaCa-2 cells, exhibited a low level of the P2X7 receptor.

Despite the lack of effect on pAkt was detected in Capan-2 cells, statins still affected apoptosis. Therefore, we evaluated two additional pathways commonly involved in regulating apoptosis in pancreatic cancer cells, Raf/MEK and NFκB pathways (Altomare DA et al 2003, Holcomb B et al 2008). We found that statins reduce NFκB activation and in the same time affected phosphorylation of Raf and MEK in Capan-2, but not in Panc-1 and MIA PaCa-2 cells. Our results support previously published data performed on nude mice. Thus, it was shown that different statins decreased the tumor growth of transplanted Capan-2 cells by an inhibition of cholesterol synthesis and by an inhibition of prenylation of signaling proteins such as Ras (Gbelcová H et al 2008). Statins have also been shown to reduce NFκB activation by inhibiting prenylation (Ortego M et al 1999; Feng Y-H et al 2005). The effects of statins on Akt in pancreatic cancer cell lines are summarized in Figure 9.

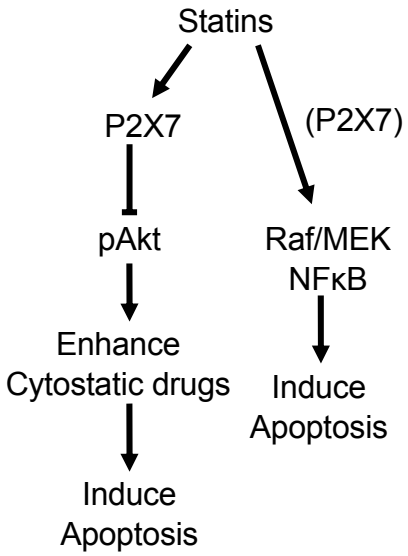

Figure 9: Summary of statin induced effects on Akt signaling in pancreas cancer cell lines.

Taken together, our data suggest that statins primarily target a functional P2X7-Akt signaling in pancreatic cancer cells. By targeting the P2X7-Akt axis, statins can sensitize pancreatic cancer cells to chemotherapeutic drugs.

4.4 Paper IV: Statin-induced depletion of pAkt correlates to changes on protein phosphatases PTEN, PHLPP and Calcineurin.

Taken together, the results in paper I and II demonstrated the rapid decrease of nuclear pAkt. Paper II and III assign a significant role of P2X7 as an important modulator of Akt activity and its downstream targets. The remaining question was how the rapid nuclear pAkt depletion was mediated. Therefore in **Paper IV** we have investigated the mechanism underlying the rapid inhibition of nuclear pAkt induced by statins that was seen in paper I and II.

PTEN has been described as a main regulator of Akt phosphorylation and it is well recognized that PTEN´s lipid phosphatase activity negatively regulates the cytoplasmic (PI3K)/Akt pathway (Perren A et al 2000, Whiteman DC et al 2002). In paper IV, we provide evidence that statin-induced effect on PTEN can be induced within a minute of treatment. Using different approaches to study the interaction between pAkt and PTEN we found that atorvastatin induced a rapid binding between the two proteins. These data suggest a causative association between PTEN and down-regulation of pAkt.

NEDD4-1 has been shown to regulate PTEN activity (Wang X et al 2007, Ahn Y et al 2008). It can mono-ubiquitinate PTEN and mediate its nuclear localization and in the nucleus PTEN can inhibit Akt (Trotman LC et al 2007). Interestingly, this is in line with our previous data showing that statins activate another ubiquitin ligase, Mdm2 (Pääjärvi G et al 2005), so a common activating mechanism might trigger the activation of both proteins Accumulation of ubiquitin in the nucleus was induced at three minutes and after five minutes nuclear levels of ubiquitin were decreased. Levels of mono-ubiquitinated PTEN is clearly increased in atorvastatin treated cells. Experiments employing NEDD4-1 and PTEN siRNA further supported an involvement of PTEN in regulating nuclear pAkt.

Furthermore, we have previously shown that statins regulate nuclear pAkt via the P2X7 purinergic receptor so we tested an involvement of P2X7 receptor in the activation of PTEN and PHLPP. As in paper II, we found that statins-induced pAkt/PTEN interaction was inhibited P2X7 antagonists, KN-62. PHLPP is a phosphatase that also has been shown to inactivate pAkt by direct dephosphorylation (Gao T et al 2005). It has previously been described that upon PHLPP activation an increased binding between PHLPP and Akt occurred (Brognard J et al 2007). Interestingly, the binding also correlated in time to the statin-induced nuclear pAkt dephosphorylation. These data are in line with a recent study showing that ATP, a natural ligand of the P2X7 receptor, modulates PTEN sub cellular localization in multiple cancer cell lines (Lobo Glenn P et al 2008). Data presented here indicate that activation of PTEN and PHLPP are downstream events to P2X7 activation.

In paper II, we have shown that atorvastatin induces increased Ca^{2+} levels in epithelial cells. Calcineurin is activated by Ca^{2+} and has been shown to be involved in the dephosphorylation and regulation of Akt (Teruel T et al 2001, Park CH et al 2008). In an attempt to understand the involvement of calcineurin we tested FK506, a selective inhibitor of calcineurin. We found that it completely prevented the effect of atorvastatin

on nuclear pAkt. Results show a rapid (one minute) increase of nuclear levels of α- and β-calcineurin.

Taken together, these data indicate that cells have the capacity to rapidly stop Akt signaling by simultaneously activating PTEN, PHLPP1 and 2, and calcineurin.

5. CONCLUSIONS

The studies presented in this thesis were aimed to elucidate the mechanism by which statins affect cancer cells. It is clear from several studies that statins can inhibit Akt in different cell lines but the mechanisms for these effects are still not well characterized. As described above, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is one of the major anti-apoptotic pathways in cells and Akt is constitutively active in many human tumors. Furtermore, many of the commonly used cytostatic drugs activate Akt in cancer cells and therefore make it an attractive target for cancer therapy. Statins, which are well-characterized and established pharmaceuticals, may thus represent a potentially new and safe class of inhibitors of Akt signaling.

In general, our results characterize the mechanism by which statins affect the Akt signaling pathway. Statins prevented Akt activation in cells stimulated by insulin or cytostatic drugs. Statins were also found to prevent nuclear localization of pAkt and kill cells pre-treated with low doses of PI3K inhibitors. Experimental design using inhibitors and siRNA approach confirmed that the effect of statin on Akt was induced in an mTOR-dependent manner. Statins did not sensitize p53-wild type cells (HepG2, A549) to p53 activating cytostatic drugs in concentrations that induce p53 stabilization. Hence, we have shown that statins may effectively potentiate apoptosis induced by p53 stabilizing anticancer drugs only in p53-deficient cells (H1299). Data indicate that an Akt-mediated sensitizing effect of statins can be counteracted by an attenuation of the p53 response.

In data not included in this thesis we found that statin induce rapid changes not only in phosphorylations of Akt but also of IRS-1, mTOR, and the p53 antagonist Mdm2, and even more rapid than previously described by our group (Pääjärvi G et al 2005) and in Paper I. Statin treatment for less than 0.5 minutes induced significant changes in levels of pmTOR Ser2448 and pERK Tyr204 in H1299 and A549 cells.

Even though statin prevented nuclear localization of pAkt induced by insulin or cytostatic drugs, the mechanism behind this was not understood. However, our findings show that nuclear level of pAkt was affected already within the first minute of statin treatment. The rapid onset of the response led us to anticipate a possible involvement of P2X receptors. Indeed, we found that both ATP and BzATP, agonists of P2X and P2X7 receptors respectively, affected nuclear levels of pAkt in a similar way as statins. We also used different experimental approaches (inhibitors, siRNA and cell lines expressing different P2X receptors) to demonstrate a significant role of the P2X7 receptors in statin induced nuclear inhibition of pAkt. In addition, we found that rapid inhibition of nuclear pAkt can not be prevented by mevalonate. The pleiotropic actions of statins may be both dependent and independent of the HMG-CoA reductase inhibition. Here we show evidence that the rapid effect of statins is induced by a HMG-CoA reductase-independent mechanism.

An interesting finding was that the effect of statin on pAkt was only detected in Panc-1 and MIA PaCa-2, but not in Capan-2 cells. This effect was induced in a p53-independent manner. We determined whether differences in responses might be explained by differences in receptor levels between the three pancreatic cancer cell

lines. We found that Capan-2 cells, in contrast to Panc-1 and MIA PaCa-2 cells, exhibited a low level of the P2X7 receptor. Moreover, we found that in Capan-2 cells statin reduce NFκB activation and in the same time affected phosphorylation of Raf and MEK. Those two pathways have been shown to regulate apoptosis in pancreatic cancer cells, by inhibition of prenylation of Ras and NFκB.

Finally, we investigated the mechanism underlying the rapid inhibition of nuclear Akt. A role of PTEN as a negative regulator was suggested. Simultaneous activation of PTEN, PHLPP1 and 2, and calcineurin was detected in the time frame where pAkt is inhibited and abolished from nucleus. A model for how PTEN and PHLPP control Akt signaling has been suggests: PTEN prevents Akt phosphorylation by inactivating the second messenger, PIP3, that activates Akt, and PHLPP inactivates Akt by direct dephosphorylation. Our data show, for the first time, that PTEN and PHLPP were activated simultaneously, bound the same substrate and apparently both participated in the down-regulation of nuclear pAkt in a rapid and coordinated action.

6. SIGNIFICANCE

Although the beneficial effects of HMG-CoA reductase inhibitors in the cardiovascular field are well established, their importance in the area of cancer therapeutics is still not well characterized. Statins are safe, well studied and established drugs. Their side effects are rare, well characterized and high levels are commonly tolerated (Vivanco et al 2002). Statins also exhibited chemopreventive effects in pancreatic and prostate cancer epidemiologically, but the mechanism behind this remains uncharacterized. We hope that our studies will increase an understanding of the chemopreventive effects. Through the experiments performed in papers I-IV, we attempted to illustrate the molecular mechanisms induced by statins. Our data showing that statins in pharmacological concentrations activate P2X7 receptors open new possibilities. P2X7 might explain many of the pleiotropic effects of statins of great clinical importance. Future work will hopefully show that statins can be used therapeutically also for non-cardiovascular interventions.

7. FUTURE PERSPECTIVES

Epidemiological studies thus indicate that statins have a strong chemopreventive effect in the prostate. Some of them suggest that aggressive prostate cancer death can be avoided. In addition, abnormally expressed P2X7 have been proposed to be an early marker for prostate cancer (Slater et al 2005). A possible role of P2X7 in this chemopreventive effect can be studied by employing molecular epidemiology and cell models.

Data presented in this thesis clearly indicate that statin-induced cellular signaling is mediated by P2X7. To further elucidate the role of P2X7, one might engage epidemiologists and perform SNP analysis. Available SHEEP samples, taken from patients with coronary infarctions and from controls can be analyzed. The idea is to analyze SNPs in the P2X7 receptor gene and some related genes and look for associations with infarctions. The goal is to provide indirect evidence that P2X7 is important for clinical effects of statins by indicating an association to cardiovascular disease in humans.

In this thesis we did not address the question if statin directly bind to P2X7 or rather trigger a cascade reaction leading to ATP release. As described in the introduction, resting cells release ATP at basal rates. This leak of cells creates an area of low ATP levels around the cells. Such area has been previously reported at the surface of platelets and Jurkat T cells (Trautman et al 2009). The authors speculate that this area mediate signals to neighboring cells about their presence. An increased ATP concentration might be triggered by statin leading to a “danger” signal leading to cell death.

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