Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

The polyphenols resveratrol and caffeic acid phenethyl ester: their influence on growth-related signaling pathways in vascular smooth muscle cells

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2006

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Frau Prof. Dr. V. M. Dirsch und von Frau Prof. Dr. A. M. Vollmar betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, am 22.09.2006

(Thomas Roos)

Dissertation eingereicht am: 29.09.2006

1. Gutachter: Prof. Dr. Verena M. Dirsch
2. Gutachter: PD Dr. Wolfgang Erl

Mündliche Prüfung am: 10.11.2006

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B INTRODUCTION

1 Background

Cardiovascular diseases are still the most prominent cause of death in the Western world. The most important exponent of cardiovascular diseases is atherosclerosis (Libby, 2002). Cardiovascular risk factors such as genetic susceptibility, high blood pressure, smoking, or high cholesterol levels result in a chronic impairment of the endothelium which then triggers a cascade of events including the recruitment and transendothelial migration of circulating leukocytes. Cytokines and growth factors are released by these immune cells, generating a highly mitogenic environment. Vascular smooth muscle cells (VSMCs), a key player during the development of atherosclerosis, start migrating towards the atherosclerotic lesion. Subsequent proliferation and deposit of extracellular matrix components of the VSMCs thus contribute to the development of the so-called neo-intima (figure 1) (Dzau *et al.*, 2002; Andres, 2004). Another burden where VSMCs play a crucial role is restenosis. This phenomenon is amongst the most common complications (in up to 60% of all cases) after percutaneous transluminal coronary-angioplasty (PTCA), vascular surgery or cardiac surgery because of mechanical injury of the blood vessel (Greenberg *et al.*, 2004). Restenosis is the result of progressive lumen narrowing caused by increased proliferation of VSMCs thereby reducing the benefit of percutaneous coronary intervention.

Hence, new therapies preventing or at least reducing the rate of atherosclerosis or restenosis are main targets of research. Current researches encompass a variety of compounds such as statins, to lower blood cholesterol, or cytostatic drugs, to prevent narrowing of the vessel. Another field of research are natural compounds, in particular those which are widely used in folk medicine or have shown to diminish cardiovascular diseases in epidemiological studies. Detailed chemical investigation of the corresponding drugs or nutritions has shown that most of them are rich in polyphenolic constituents.

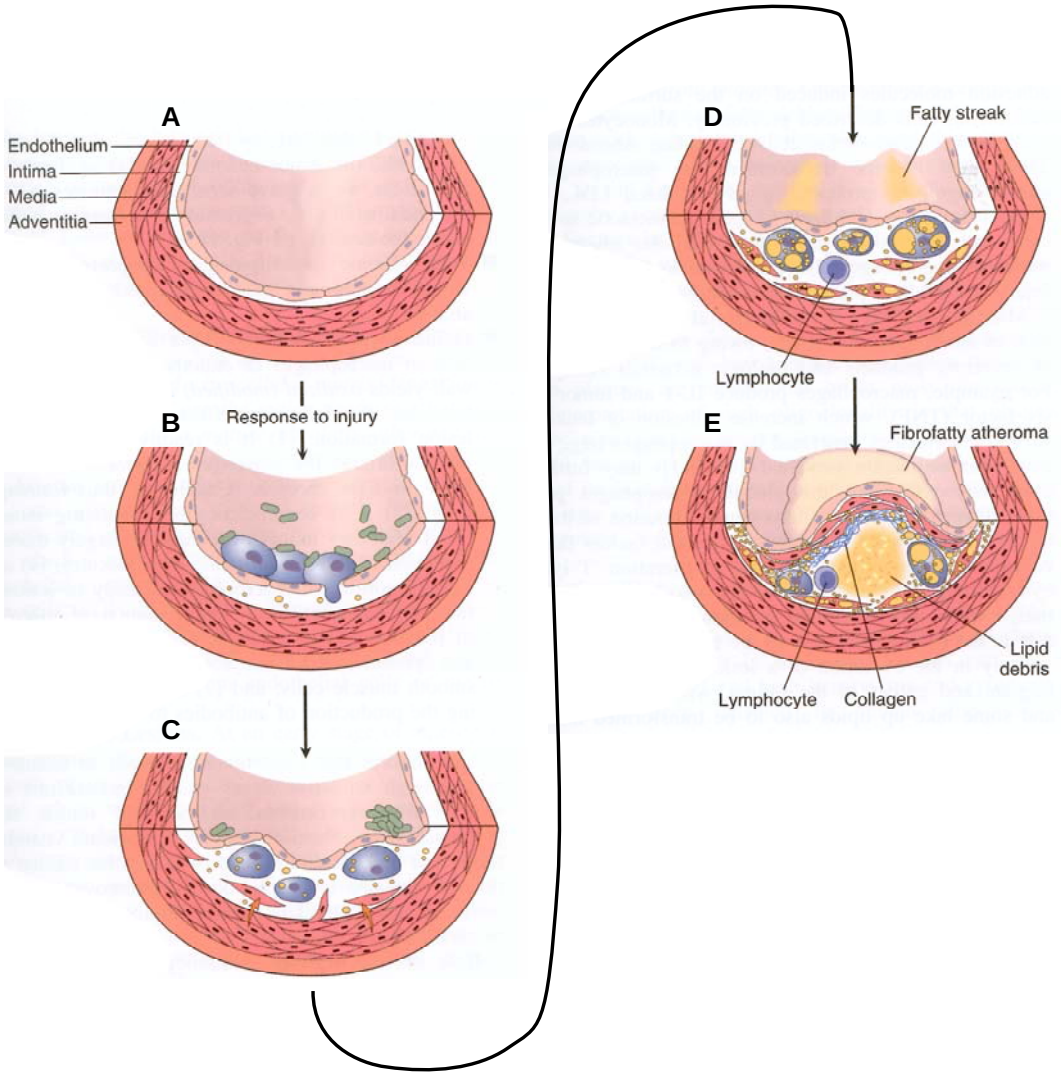


Figure 1. Processes in the response to injury hypothesis. **A**, normal vessel wall. Chronic endothelial impairment, hyperlipidemia, hypertension, and smoking leads to **B**, endothelial dysfunction (e.g. increased permeability, leukocyte adhesion). **C**, migration of smooth muscle cells from the media into the intima. **D**, Activation and transformation of monocytes into macrophages. After incorporation of lipid the macrophages become so-called foam cells eventually resulting in macroscopically apparent fatty streaks. **E**, intimal smooth muscle cells start proliferating and depositing extracellular matrix. This process together with foam cell-derived lipid debris converts the fatty streaks into fibrofatty atheroma (Schoen F.J. and Cotran R.S., 1999).

Two candidates worth being investigated are resveratrol (RV; *trans*-3,5,4'-trihydroxystilbene), and caffeic acid phenethyl ester (CAPE; *trans*-3-(3,4-dihydroxyphenyl)propenoic acid phenethyl ester).

Resveratrol e.g. has been suggested to account for the so-called "French paradox", because it is a major component of red wine (Poussier *et al.*, 2005) (for more details see chapter 4). And CAPE, the major constituent of propolis (see also section 5) has been shown to reduce restenosis in a rat model of vascular injury (Maffia *et al.*, 2002).

2 Aim of the work

Objective of the present study was to investigate the effects of resveratrol (RV) and CAPE on VSMC hypertrophy and hyperplasia respectively. Primary rat aortic smooth muscle cells are widely used and accepted as a model in cardiovascular research. So, we used these cells to gain insight into the molecular signaling of VSMCs upon treatment with the above mentioned natural compounds. Since a recent study of our group has shown that RV suppresses angiotensin II induced hypertrophy in rat VSMCs (Haider *et al.*, 2002), angiotensin II (Ang II, refer to chapter 6) and epidermal growth factor (EGF, see also section 7) were applied to investigate the underlying mechanism of resveratrol on inhibition of hypertrophy. Platelet-derived growth factor-BB (PDGF-BB) (a detailed description can be found in chapter 10) was used to examine the properties of CAPE on VSMC proliferation.

The following two questions should be answered:

1. Elucidation of signal transduction processes: what proteins are involved in the RV-mediated Akt inhibition in Ang II or EGF-activated VSMCs?
2. Is CAPE capable of inhibiting PDGF-BB-induced proliferation of VSMCs? If so, what signaling pathways are involved?

3 Vascular smooth muscle cells (VSMCs)

3.1 VSMCs in arterial narrowing

In healthy arteries, VSMCs are fully differentiated and non-proliferating and belong to the media of the vessel wall (composition of the vessel see figure 1). However, if the inner arterial wall is damaged, VSMCs re-enter the cell cycle by means of cytokine and growth factor release from recruited platelets and impaired endothelial cells. This effect starts a few hours after injury of the vessel wall, e.g. after balloon angioplasty. Migration of the VSMCs into the intima begins at the fourth day, where they proliferate for another two weeks. At the end, extracellular matrix (ECM) is increasingly deposited which leads to fortified narrowing of the vessel (Braun-Dullaeus *et al.*, 1998). Initially, this stenosis is compensated by progressive dilation of the vessel, but sustained ECM deposit finally obstructs blood flow and leads to the typical symptoms of atherosclerosis (Libby, 2002) such as angina pectoris or gangrene.

3.2 Cultured VSMCs

Culturing VSMCs leads to an altered phenotype compared to their *in vivo* counterparts. While *in vivo* smooth muscle cells show a contractile shape, cultured SMCs change to a more synthesizing phenotype, which is characterized by enhanced proliferation and ECM depositing (Shanahan and Weissberg, 1998). This behavior is similar to that of VSMCs during neointima formation and therefore a suitable model for cardiovascular research.

4 Resveratrol (RV)

4.1 Discovery, sources and biological function

Resveratrol can be found in grapes, peanuts and a few other fruits and vegetables. It was first isolated from the roots of Japanese knotweed *(Polygonum cuspidatum)* by Nonomura *et al*. in 1963. This herb is used in Asian folk-medicine against suppurative dermatitis, gonorrhea and hyperlipidemia (NONOMURA *et al.*, 1963). Major interest in resveratrol came up with its discovery in red wine in 1992 (Siemann and Creasy, 1992). Since it is present in grape berry skins but not in grape flesh, white wine contains only small amounts of resveratrol. Concentrations in fresh grape skin vary from 50 to 100 mg/g (Soleas *et al.*, 1997). Since resveratrol is a phytoalexin, this compound is produced in response to UV-irradiation, exposure to ozone or pathogen attack. Grapes (*Vitis vinifera*) synthesize resveratrol in response to fungal infections. Thus, it is more abundant in grapes grown in cooler, humid climates where fungal infections are more frequent.

4.2 Features

By its discovery in red wine 1992, resveratrol gained increasing interest and many of studies have been performed, revealing an increasing number of biological properties. The following passage will focus on the features of major interest for this study.

4.2.1 Anti-inflammatory properties

One important family of enzymes involved in inflammation are cyclooxygenases (COX). Resveratrol has been shown to interfere with COX, however alterations of COX gene expression and activity by resveratrol differ in diverse models (Jang *et al.*, 1997; Chow *et al.*, 2005). Anyhow, anti-inflammatory properties of resveratrol were confirmed *in vivo*, since carrageenan-induced paw edema was suppressed by resveratrol in both the acute and the chronic phase (Jang *et al.*, 1997). In addition to COX, inhibition of the 5-lipoxygenase and the

15-lipoxygenase pathway was also demonstrated in several models (Soleas *et al.*, 2001).

4.2.2 Cell cycle

In several cell lines including VSMCs, resveratrol exhibits antimitogenic properties. Proliferation and DNA synthesis was shown to be inhibited dose-dependently (Oak *et al.*, 2005; Haider *et al.*, 2003; Chao *et al.*, 2005). Referring to this, *in vitro* experiments investigating enzyme activity of ribonucleotidreductase (Matsuoka *et al.*, 2004; Fontecave *et al.*, 1998) as well as DNA-polymerase α and δ (Locatelli *et al.*, 2005), revealed that resveratrol acts as an inhibitor of these enzymes. Resveratrol showed various effects on other important cell cycle proteins such as the cyclins, $p21^{Cip1}$ or the retinoblastoma protein (Rb) (Chow *et al.*, 2005; Poussier *et al.*, 2005; Haider *et al.*, 2003; Adhami *et al.*, 2001; Hsieh *et al.*, 2002).

4.2.3 Cardiovascular effects

Resveratrol has been shown in several studies to prevent platelet aggregation (Delmas *et al.*, 2005; Olas and Wachowicz, 2005). Furthermore, tumor necrosis factor α (TNF-α)-induced expression of the adhesion molecules ICAM-1 and VCAM-1 as well as adhesion of THP-1 monocytic cells to human umbilical vein endothelial cells has been demonstrated to be reduced by resveratrol (Ahn *et al.*, 2000). It has been reported that resveratrol leads to an increase in eNOS activity, protein and mRNA expression (Wallerath *et al.*, 2002). More recently, Kaga *et al.* recently provided evidence that resveratrol is capable of enhancing neo-vascularization in the infarcted rat myocardium (Kaga *et al.*, 2005).

4.2.4 Bioavailability

To evaluate the positive effects of moderate wine consumption and to review the *in vivo* efficiency bioavailability studies have been performed. Some red wine polyphenols, including *trans*-resveratrol, have been shown to be highly absorbed but also rapidly and extensively metabolized through glucuronidation or sulfatation in the liver (Goldberg *et al.*, 2003; Meng *et al.*, 2004; Vitaglione *et al.*, 2005). The half-life of RV is ~8-14 minutes for the unchanged molecule

(Baur and Sinclair, 2006). On the other hand, Bertelli *et al.* suggested an accumulation of resveratrol in different organs after prolonged oral administration to rats (Bertelli *et al.*, 1996), and Vitaglione *et al.* investigated absorption of resveratrol from moderate consumption of red wine (300-600 ml). They could show that resveratrol can actually be absorbed even after moderate wine consumption (Vitaglione *et al.*, 2005). So far, the existing data on the bioavailability of resveratrol is contradictory and confusing. The various different settings within all the studies raise some doubts about the concentration that are achievable *in vivo* (Baur and Sinclair, 2006). Therefore, it is difficult to connect the well-known beneficial effects of RV obtained from *in vivo* or cell-based studies to potential effects expected after ingestion of resveratrol-containing foods.

5 Caffeic acid phenethyl ester (CAPE)

5.1 Discovery, source and biological function

Caffeic acid phenethyl ester (CAPE) was first described by Grunberger *et al.* in 1988 as the major active element of propolis (Grunberger *et al.*, 1988), a resinous substance collected from honey bees. The name propolis is derived from the Greek syllables *pro* which means “in front of” and *polis* which means “city” and stands for a material to cover hive walls, fill gaps and embalm killed invaders, briefly a substance to defend the bee-hive. Propolis is an old remedy which has been used at least since 300 BC. Until now, over 180 compounds, mainly polyphenols, have been identified from propolis.

5.2 Features

CAPE was isolated from propolis in 1988 (Grunberger *et al.*, 1988). The subsequent discovery of its preferential cytotoxicity on tumor cells was the matter for further studies, revealing numerous biological properties. Therefore this paragraph will give a short overview of distinct features of CAPE.

5.2.1 Antioxidative properties

CAPE has shown its antioxidative activities in different biological systems and *in vitro* (Ozguner *et al.*, 2005; Oktem *et al.*, 2005; Hsu *et al.*, 2005). The antioxidative activity of CAPE was first described by Sud'ina *et al*. (Sud'ina *et al.*, 1993) showing that CAPE is an antioxidant which is also able to interfere with the arachidonic acid pathway *via* inhibition of 5-lipoxygenase (5-LOX) thus reducing inflammation.

5.2.2 Anti-inflammatory and chemopreventive properties

CAPE has been shown to inhibit 5-lipoxygenase (Sud'ina *et al.*, 1993). Moreover several studies with CAPE demonstrate cyclooxygenase (COX) inhibition (Lee *et al.*, 2004; Ye *et al.*, 2004). Inhibition of the nuclear transcription factor κB (NF-κB) is the best investigated activity of CAPE (Natarajan *et al.*, 1996; Hishikawa *et al.*, 2005; Lee *et al.*, 2004; Ye *et al.*, 2004). NF-κB is not only important in inflammation, as e.g. the *cox2* promotor contains NF-κB binding sites (Lee *et al.*, 2004) but it has been shown to be constitutively activated in several types of cancer cells (Ye *et al.*, 2004). Therefore it is not surprising that several studies have shown that CAPE is able to induce apoptosis in different cancer cell lines (Orsolic *et al.*, 2004; Chen *et al.*, 2004).

5.2.3 Cardiovascular effects

Maffia *et al.* could give evidence that CAPE is able to reduce occurring restenosis after balloon angioplasty in a rat model (Maffia *et al.*, 2002). Other studies demonstrated that CAPE causes relaxation of the phenylephrin or KCl pre-contracted rat thoracic aortic ring (Cicala *et al.*, 2003), and that CAPE attenuates atherosclerosis development in apolipoprotein E-deficient mice (Hishikawa *et al.*, 2005). Moreover CAPE was able to inhibit *chlamydophila pneumoniae*-induced ICAM-1 upregulation in human aortic endothelial cells (Vielma *et al.*, 2003).

5.2.4 Effects on proliferation

CAPE exhibits anti-proliferative properties in different cell types including vascular smooth muscle cells (Wang *et al.*, 2005; Liao *et al.*, 2003; Zheng *et al.*, 1995). It was shown to inhibit angiogenesis in chick embryo chorioallantoic membranes (Song *et al.*, 2002). This result is consistent with findings showing CAPE to reduce the vascular endothelial growth factor (VEGF) production and expression of matrix metalloproteinase (MMP)-2 and -9 in CT26 cell culture (Liao *et al.*, 2003).

5.2.5 Bioavailability

Until today only little is known on how effectively CAPE is absorbed after oral supplementation. However there are some recent studies where CAPE has been administered orally. Celli *et al.* developed and validated a new analytical method to determine CAPE in rat plasma and urine. They showed that CAPE is rapidly absorbed and excreted in urine both as unmodified molecule and as glucuronide conjugate although in a low amount compared to the administered dose (Celli *et al.*, 2004). Effects of CAPE on the immune system in mice suggest an immunemodulatory capacity of CAPE when given orally (Park *et al.*, 2004; Orsolic *et al.*, 2006). CAPE was shown to be capable of attenuating the development of atherosclerosis in ApoE null mice after oral application (Hishikawa *et al.*, 2005). Up to now, the existing data on the bioavailability of CAPE is difficult to interpret. CAPE has been shown to be quickly absorbed but it is also rapidly metabolized and excreted. The existing animal studies, though, show that an oral intake of CAPE is associated with biological effects *in vivo* that can be demonstrated also *in vitro*.

6 Angiotensin II

6.1 History

More than 50 years ago, synthesis and pharmacologic properties of angiotensin II were described for the first time. For a long time angiotensin II was only regarded an important and potent vasoconstrictor. It was in the early 1990s that pro-inflammatory and proliferative properties were found for angiotensin II. Since then rapid progress was made in the elucidation of the underlying mechanisms of these findings (Alexander and Dzau, 2000).

6.2 Structure and metabolism

Angiotensin II is an octapeptide hormone that can be produced systemically *via* the renal renin-angiotensin system as well as locally by the tissue renin-angiotensin system (Touyz and Berry, 2002).

6.2.1 The renin-angiotensin system:

Angiotensinogen, a hepatic-derived α-globulin, is cleaved at the N-terminus by means of renal-derived renin. In the lungs, the resulting decapeptide angiotensin I is then converted into angiotensin II by action of the dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE).

The latter step can also be catalyzed by several enzymes different from ACE, such as chymase, carboxypeptidase and cathepsin G. But although these enzymes are present in atherosclerotic lesions, the affinity of angiotensin I to ACE is higher, suggesting that angiotensin II generation in atherosclerotic vessels depends mainly on ACE (Schmidt-Ott *et al.*, 2000).

Angiotensin I can be transformed further to the heptapeptide angiotensin II (1-7) by tissue endopeptidases. Angiotensin II (1-7) seems to be a naturally occurring antagonist of angiotensin II actions (Touyz and Berry, 2002).

Subsequent degradation of angiotensin II to angiotensin III and angiotensin IV is performed by aminopeptidases (Touyz and Berry, 2002).

6.2.2 The tissue angiotensin:

Generation of angiotensin II is not limited to the renal renin-angiotensin system as all components are expressed in the vessel wall (Schmidt-Ott *et al.*, 2000).

Angiotensinogen mRNA was found in VSMCs of healthy arteries and it has been shown that the levels increased in both the media and the neointima after balloon angioplasty in rats (Rakugi *et al.*, 1993). Renin mRNA and protein were detected predominantly in the media and here, too, the levels rose after balloon injury (Iwai *et al.*, 1997). ACE seems to prevail in endothelial cells, but in atherosclerotic lesions it can be detected also in macrophages (Fukuhara *et al.*, 2000).

Altogether, there is evidence that these tissue renin-angiotensin systems may play an important role in both local regulation of blood flow and pathogenesis of cardiovascular disease (Weiss *et al.*, 2001; Touyz and Berry, 2002).

6.3 Receptors

Angiotensin II mediates its effects through G protein-coupled receptors (GPCRs). So far four receptors have been identified of which AT_1 and AT_2 play the most important role. The role of AT_3 and AT_4 has not been entirely elucidated yet (Touyz and Berry, 2002).

Most vascular effects of angiotensin II are mediated *via* the AT_1 receptor (AT_1-R). This seven-transmembrane glycoprotein, composed of 359 amino acids, activates phospholipase C (PLC) *via* a heterotrimeric G_q protein (Touyz and Berry, 2002). The AT_1-R is localized mainly in VSMCs, whereas levels in the adventitia are low and endothelial cells seem not to contain significant amounts (Allen *et al.*, 2000; Zhuo *et al.*, 1998). Interestingly, the density of AT_1-R is increased in the media of damaged blood vessels compared to that of healthy animals (Yang *et al.*, 1998).

The AT_2-R is a seven-transmembrane GPCR, too, but shares only little homology with the AT_1-R (approx. 32%). It consists of 363 amino acids and is expressed predominantly in fetal tissues. After birth the levels decrease very

fast and are restricted to certain organs such as the brain, adrenal medulla, kidney, uterus, and ovary (Johren *et al.*, 2004). Only 10% of the total angiotensin receptors in healthy vessels are of the AT_2-type (Schmidt-Ott *et al.*, 2000) and are mainly localized in adventitia and endothelial cells, but not in VSMCs (Zhuo *et al.*, 1998; Wang *et al.*, 1998). There is evidence that levels of the AT_2-R, in contrast to AT_1-R, are not altered in atherosclerotic vessels (Yang *et al.*, 1998).

Until now, the main function of the AT_2-R is believed to mediate antagonistic effects compared to that of the AT_1-R (Allen *et al.*, 2000).

6.4 Physiology

Angiotensin II causes VSMC contraction within seconds. This effect is mediated by activation of PLC-β1 *via* the G_q-coupled AT_1-R and subsequent increase of intracellular Ca^{2+} levels (Touyz and Berry, 2002). Ca^{2+} associates with calmodulin, resulting in the activation of the myosin light chain kinase, a crucial requirement for contraction (Morano, 1992). The Ca^{2+}-increase upon angiotensin II stimulation is biphasic. First, PLC-β1 hydrolyses 4,5-phosphorylated phosphatidylinositol (PtdIns) generating inositol triphosphate (IP_3) and diacylglycerol (DAG). Intracellular Ca^{2+} is mobilized by IP_3 from the sarcoplasmic reticulum, leading to a rapid and transient increase in Ca^{2+} levels. The more sustained second wave results from transmembrane Ca^{2+} influx. In addition to IP_3-mediated mobilization of intracellular Ca^{2+} and influx of extracellular Ca^{2+}, tyrosine kinase-dependent increase of intracellular Ca^{2+} has been suggested (Touyz and Berry, 2002).

DAG, the other second messenger generated by PLC, also contributes to vasoconstriction. Together with Ca^{2+} and phosphatidylserine, it activates protein kinase C (PKC), a serine/threonine kinase existing in several isoforms. PKC leads to activation of the Na^+/H^+ exchanger, thereby resulting in intracellular pH shift towards alkaline conditions. This alkalization has been shown to induce vasoconstriction by increasing intracellular Na^+ and Ca^{2+} levels and sensitizing the contractile apparatus (Touyz and Berry, 2002).

Finally, angiotensin II regulates blood pressure and plasma volume by stimulation of renal Na^+ and water resorption, aldosterone release, sympathetic nervous system and thirst response (Touyz and Berry, 2002).

6.5 Pathophysiology

The pathologic activities of angiotensin II resulting in atherosclerosis are not restricted to VSMCs but involve all components of the vessel wall. This chapter will first give a brief summary of angiotensin II actions in the vascular wall in general. Afterwards, the effects of angiotensin II in VSMC will be discussed in more detail.

6.5.1 Angiotensin II and the vessel wall

Over the last century, several theories have emerged to account for the development, progression, and complication of atherosclerosis. The lipid hypothesis proposed by Anitschkow and Chalatow in 1913 (Nil, 1913) attributed atherosclerosis to gradual lipid accumulation in the arterial wall. In the thrombogenic hypothesis, growth of atherosclerotic plaques results from gradual incorporation of luminal thrombi into the arterial wall (Weissberg and Rudd J, 2002). The response to injury hypothesis supports the notion of atherosclerosis as a protective response to various factors causing endothelial injury (Ross, 1999). Currently, inflammation is being recognized as pivotal in the pathogenesis of atherosclerosis (Tiong and Brieger, 2005). In experimental settings many features of atherosclerosis can be mimicked by angiotensin II.

Furthermore, angiotensin II triggers both inflammation and oxidative stress (Weiss *et al.*, 2001).

The hormone is crucially involved in the generation of reactive oxygen species (ROS) in the vasculature. The most important source of ROS in the vessel is NAP(P)H oxidase (NOX). Other relevant sources are xanthine oxidase, lipoxygenases (LOX), and cytochrom P_{450} monooxygenase (Touyz and Berry, 2002). Increased, angiotensin II-induced ROS generation contributes to endothelial dysfunction and oxidation of low-density lipoprotein (LDL).

Moreover, angiotensin II increases the expression of cell adhesion molecules on endothelial cells and VSMCs thereby enhancing leukocyte infiltration through the vessel wall (Schmidt-Ott *et al.*, 2000). Last but not least, angiotensin II is capable of inducing apoptosis in endothelial cells. This may contribute to damage in the endothelial monolayer (Schmidt-Ott *et al.*, 2000).

6.5.2 Angiotensin II and VSMCs

Effects on intracellular signaling

Angiotensin II provokes plenty of actions by activation of AT_1-Rs in VSMCs ranging from phosphorylation and activation of various kinases and phospholipases to transactivation of receptor tyrosine kinases (RTKs) (Touyz and Schiffrin, 2000). In the following paragraph the pathways important for this study are discussed.

Effects on VSMC function

Protein synthesis and cellular hypertrophy in cultured VSMCs is one effect of angiotensin II stimulation (Berk *et al.*, 1989; Geisterfer *et al.*, 1988). However, in the presence of other growth factors hyperplasia can be induced by angiotensin II. Additionally angiotensin II has been shown to provoke VSMC migration in transwell-culture chambers (Xi *et al.*, 1999).

Beside these direct effects, angiotensin II can act indirectly inducing the production of various growth factors, cyto- and chemokines, including macrophage chemoattractant protein 1 (MCP-1), interleukin 6 (IL 6), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF). All these molecules, except TGF-β which induces collagen synthesis, enhance VSMC proliferation and migration and augment inflammation (Schmidt-Ott *et al.*, 2000).

Furthermore, angiotensin II induces changes in extracellular matrix (ECM) composition. Non-proliferating, contractile VSMCs synthesize only little amounts of ECM. During vascular remodeling, VSMCs change their phenotype towards a

proliferative, synthesizing one, thereby increasing production of fibronectin, collagen and elastin. Interestingly, these changes in the extracellular environment promote proliferation and migration (Schmidt-Ott *et al.*, 2000).

7 Epidermal growth factor receptor (EGF-R) transactivation

The EGF-R can be activated by direct ligand binding and by transactivation through other pathways, including those triggered by cytokine receptors, ion channels and GPCRs (Breitling and Hoeller, 2005). Transactivation of the EGF-R through the AT_1-R has been suggested to be a pivotal step in angiotensin II signaling (Voisin *et al.*, 2002; Wolf, 2005; Yang *et al.*, 2005). To focus on signaling downstream of EGF-R transactivation in several experiments epidermal growth factor (EGF) was used to activate VSMCs.

7.1 EGF-Receptors

The EGF-receptor (EGF-R, also termed HER1/ErbB-1) was the first receptor tyrosine kinase (RTK) to be discovered in 1978 (Carpenter *et al.*, 1978). It belongs to the EGF-R subfamily of RTKs.

The EGF-R is a transmembrane glycoprotein consisting of 1186 amino acids. The intracellular domain can be subdivided into three domains: the juxtamembrane domain, which serves as a site for feedback attenuation by protein kinase C (PKC) and extracellular-signal regulated kinase 1/2 (Erk1/2). It is followed by the kinase domain which in turn is succeeded by the carboxy-terminal tail that has autoinhibitory function by serving as an inhibitory substrate for the kinase domain. Autophosphorylation of the EGF-R is a prerequisite for kinase activation. The resulting phospho-sites serve as docking-sites for several signaling and scaffold molecules containing phospho-tyrosine binding domains (Dreux *et al.*, 2005). Upon ligand binding to the extracellular domain, the conformation of the receptor is changed hence leading to the exposure of receptor-receptor interaction site. This results in the dimerization of two ligand-occupied EGF-Rs. All EGF-R family members are capable of building dimers.

The dimerization facilitates intermolecular autophosphorylation resulting in full tyrosine kinase activity (Dreux *et al.*, 2005).

7.2 Transactivation by the AT_1-Receptor

EGF-R transactivation in VSMCs upon stimulation of the AT_1-R has been shown to be essential for protein synthesis (Voisin *et al.*, 2002). It has further been demonstrated to be linked to the activation of extracellular-signal regulated kinase 1/2 and p38 MAPK (Eguchi *et al.*, 2001; Frank *et al.*, 2001), the serine/threonine kinase Akt and p70 S6 kinase ($p70^{S6K}$) (Eguchi *et al.*, 1999b).

Due to the rapid phosphorylation of the EGF-R as well as a result of lack of EGF-R ligands in conditioned medium, transactivation of the EGF-R was believed to be mediated solely by intracellular events (Daub *et al.*, 1997). Various pathway have been considered. It was shown that in VSMCs Ang II stimulation led to transactivation of the EGF-R and subsequently to its association with the adaptor proteins Shc and Grb2. This reaction was mimicked by using by a calcium ionophore and completely inhibited by an intracellular Ca^{2+} chelator (Eguchi *et al.*, 1998). Proline-rich tyrosine kinase 2 (Pyk 2), a calcium dependent tyrosine kinase, was demonstrated to be involved in the transactivation cascade. Ang II leads to phosphorylation of Pyk2 which in turn is able to activate EGF-R associated c-Src thereby causing transactivation (Eguchi *et al.*, 1999a). The Ang II induced c-Src activation was shown to be crucially dependent on the generation of reactive oxygen species (ROS) (Ushio-Fukai *et al.*, 2001a). To summarize, concerning angiotensin II signaling in VSMCs, the tyrosine kinase c-Src, Ca^{2+}-dependentent activation of Pyk2 and reactive oxygen species have been considered to be essential for EGF-R transactivation.

However, there is evidence that transactivation includes also extracellular events. Activated AT_1-receptor on vascular smooth muscle cells causes the release of intermediatory signaling molecules, such as PKC and reactive oxygen species (ROS). These in turn induce a metalloproteinase-dependent

cleavage of heparin-binding epidermal growth factor (HB-EGF), which proceeds to activate the EGF receptor and its downstream signaling pathways (Prenzel *et al.*, 1999; Shah and Catt, 2003; Ushio-Fukai *et al.*, 2001a). This process has been termed a triple membrane passing signal event (TMPS) (Harris *et al.*, 2003). As apparent from its name, HB-EGF possesses not only EGF-like properties but also a heparin-binding domain that binds to heparan sulfate side chains in heparan sulfate proteoglycanes which serve as co-activators of EGF-R. Noteworthy, HB-EGF has been linked to hyperplasia and atherosclerosis in VSMCs (Raab and Klagsbrun, 1997).

8 The scaffolding protein Grb2-associated binder 1 (Gab1)

A major challenge of research is to determine how the initial activation of growth factor receptors leads to multiple downstream signaling cascades. As mentioned above, the tyrosine phosphorylation of the receptors generates docking sites for several molecules containing Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains. These proteins in turn can be divided in two categories, enzymes or transcription factors (e.g. PLCγ, Stats) or adaptors (e.g. Grb2). The latter typically lack intrinsic catalytic activity but they associate with one ore more enzymes.

There are various signaling cascades using 'scaffolding adaptors'. These scaffolding adaptors contain a membrane-targeting sequence (e.g. pleckstrin homology (PH) domain) and multiple tyrosine phosphorylation sites that serve as docking sites for additional SH2- or PTB-domain containing proteins (Gu and Neel, 2003). Unlike adaptors such as Grb2, scaffolding proteins can be targeted to specific membrane lipids. Scaffolding adaptors assemble multimeric signaling complexes due to their ability to multiple signal relay proteins. Although only little is know about the scaffolding protein so far, they seem to be some kind of amplifier for intracellular signaling (Rodrigues *et al.*, 2000; Gu and Neel, 2003).

8.1 Gab1 – identification and function

Gab proteins encompass a family of scaffolding proteins which are conserved from worms to mammals. The family of proteins consists of Gab1, Gab2, Gab3, *Drosophila* Dos, and *Caenorhabditis elegans* Soc1. Concerning this study the following paragraphs will focus on Gab1.

Gab1 was identified in a search for Grb2 SH3-domain binding proteins and it is expressed ubiquitously (Holgado-Madruga *et al.*, 1996). It contains an N-terminal PH domain, proline-rich motifs and multiple potential tyrosine and serine/threonine phosphorylation sites.

Gab1 is recruited to activated RTKs mostly *via* Grb2. The role of the PH domain in Gab recruitment is best understood for EGF-R signaling. Briefly, Gab1 is initially targeted to the EGF-R *via* Grb2, whereupon it becomes tyrosine phosphorylated and interacts with the p85 subunit of PI3K (Rodrigues *et al.*, 2000). This leads to activation of PI3K and the generation of 3-phosphoinositide lipids. The Gab1 PH domain then binds to these lipids, resulting in Gab1 retention near the receptor and more sustained signaling. In agreement with this model, the PH domain is required for maximal EGF-induced Gab1 tyrosine phosphorylation and activation of downstream effectors (Rodrigues *et al.*, 2000).

9 SH2-containing protein phosphatase 2 (Shp-2)

Several signaling pathways are regulated *via* tyrosine phosphorylation. This phosphorylation can result in altered enzyme activity, assembly of signaling complexes, and protein localization which in turn trigger downstream signaling events. The dimension of tyrosine phosphorylation can be regulated by two types of enzymes: protein-tyrosine kinases (PTKs), which catalyze phosphorylation, and protein-tyrosine phosphatases (PTPs), which direct dephosphorylation. Elucidation of these processes are major aims of current signal transduction research.

Src homology-2 (SH2) domain-containing phosphatases (Shps) are a small, highly conserved subfamily of protein-tyrosine phosphatases which are expressed in both vertebrates and invertebrates. There are two vertebrate Shps – Shp-1 and Shp-2. *Drosophila* and *Caenorhabditis elegans* each have one Shp ortholog, Corkscrew (Csw) and Ptp-2, respectively. Shp-2 is expressed ubiquitously in mammalian cells, whereas Shp-1 expression is more restricted with highest levels in hematopoietic cells (Neel *et al.*, 2003). Shp-2 contains two N-terminal SH2 domains (N-SH2 and C-SH2), a PTP domain and a C-terminal tail. The basal activity of Shp-2 is low owing to allosteric inhibition of its PTP domain by the N-terminal SH2 domain. Upon Shp-2 binding to Gab1 protein, basal inhibition is relieved, resulting in strong activation.

9.1 Function of Shp-2

In most RTK signaling pathways, Shp-2 is required for full activation of the Erk MAP kinase pathway. Although Shp-2 clearly regulates Ras/Erk activation, genetic and biochemical evidence indicate additional roles (Neel *et al.*, 2003). Shp-2 mutant fibroblasts have shown an increased JNK activation in response to various stress stimuli (Shi *et al.*, 1998). Moreover, in some RTK (e.g. PDGFR and IGFR) pathways, Shp-2 is required for PI3K activation (Wu *et al.*, 2001; Zhang *et al.*, 2002). In others (e.g. EGFR signaling), Shp-2 negatively regulates PI3K activation by dephosphorylating PI3K-binding sites on Gab1 (Zhang *et al.*, 2002).

10 Platelet-derived growth factor (PDGF)

Platelet-derived growth factors (PDGFs) were identified in 1974 as constituent of whole blood serum that was able to stimulate vascular smooth muscle cell proliferation (Ross *et al.*, 1974). PDGF was initially purified from human platelets and it was one of the first growth factors to be characterized. In addition, it has, to some extend, served as a model for growth factor studies in general.

10.1 The isoforms – structure and activation

The PDGF family consists of four different proteins, PDGF-A, PDGF-B, PDGF-C, and PDGF-D, encoded by four different genes. About thirty years ago, PDGF-A and PDGF-B, the classical PDGF chains, were discovered. PDGF-A consists of 196 or 211 amino acid residues due to alternative splicing and PDGF-B is 241 amino acids in length. PDGF-C and PDGF-D were discovered more than 20 years later (Li *et al.*, 2000; LaRochelle *et al.*, 2001) and are composed of 345 and 370 amino acids, respectively. The PDGF chains form homo- and heterodimers, of which five different dimeric isoforms have been described so far: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. A highly conserved growth factor domain, referred to as the PDGF/VEGF homology domain is present in each of the four PDGF chains. This domain consists of approximately 100 amino acids and is characterized by eight highly conserved cysteine residues. Two of the cysteines (the second and fourth) are involved in disulfide bonds between the two subunits in the PDGF dimer, and the other six are engaged in intrachain cysteine bonds (the first pairs with the sixth, the third with seventh and the fifth with the eighth). X-ray crystallography of the structure of PDGF-BB revealed that the two subunits of the growth factor dimers are arranged in an anti-parallel fashion (Oefner *et al.*, 1992). The subunits consist of a knot-like structure in which one of the intramolecular disulfides reaches through the cavity formed by the two other ones and the interjacent sequences (Murray-Rust *et al.*, 1993).

All four PDGFs are synthesized as precursor molecules and proteolytic processing is necessary for their activation and biological function. However, there is an important difference in the mechanism. In the classical PDGFs A and B the amino-termini are intracellularly processed in the *trans*-Golgi network during protein maturation and secretion. In contrast, PDGF-C and PDGF-D are cleaved by extracellular proteases to be activated (Fredriksson *et al.*, 2004).

10.2 PDGF receptors

The α- and β-receptors have molecular sizes of ~170 and 185 kDa, respectively. Extracellularly, each receptor contains five immunoglobulin-like domains, whereas intracellularly there is a tyrosine kinase domain that contains a characteristic inserted sequence without homology to kinases. The five dimeric isoforms of PDGF show different capabilities of binding to the two existing PDGF receptors (PDGF-Rs). PDGF-A, PDGF-B and PDGF-C bind to PDGF-Rα while PDGF-Rβ can be bound and activated by PDGF-B and PDGF-D (Fredriksson *et al.*, 2004). Given that the PDGF isoforms are dimeric they bind two receptors simultaneously and thus dimerize the receptors upon binding. From this results a receptor binding specificity of the five dimeric isoforms as shown in figure 2. Dimerization of the PDGF receptors leads to their autophosphorylation. This phosphorylation serves two important functions: on one hand, phosphorylation of conserved tyrosine residues inside the kinase domain (Tyr849 in the α-receptor and Tyr857 in the β-receptor) results in an increase in the catalytic activities of the kinases. On the other hand, autophosphorylation of tyrosine residues outside the kinase domain generates docking sites for SH2 domain containing signal transduction molecules. The SH2 domain is a conserved motif of ~100 amino acids that can bind phosphorylated tyrosines in a specific environment (Pawson and Scott, 1997). A large number of SH2 domain containing proteins have been shown to bind to the PDGF receptors. Some of these molecules are themselves enzymes, such as PI3-K, PLC-γ, the Src family of tyrosine kinases or the phosphotyrosine phosphatases Shp-2. Other molecules such as Grb2, Shc, and Crk lack enzymatic activity and serve as adaptor molecules, linking the receptor to downstream catalytic molecules.

It has been shown that PDGF initiates stimulatory and inhibitory signals often in parallel (Heldin, 1997). Furthermore, there is evidence that this phenomenon is owing to activation of different receptors. While PDGF-Rβ more often mediate positive chemotactic and mitogenic signals, PDGF-Rα may mediate either positive or negative signals, depending on the cell type (Vassbotn *et al.*, 1992;

Yokote *et al.*, 1996; Siegbahn *et al.*, 1990; Koyama *et al.*, 1992; Koyama *et al.*, 1994). In VSMCs it has been shown that PDGF-BB exerts positive chemotactic and proliferative effects (Bornfeldt *et al.*, 1994; Kundra *et al.*, 1994).

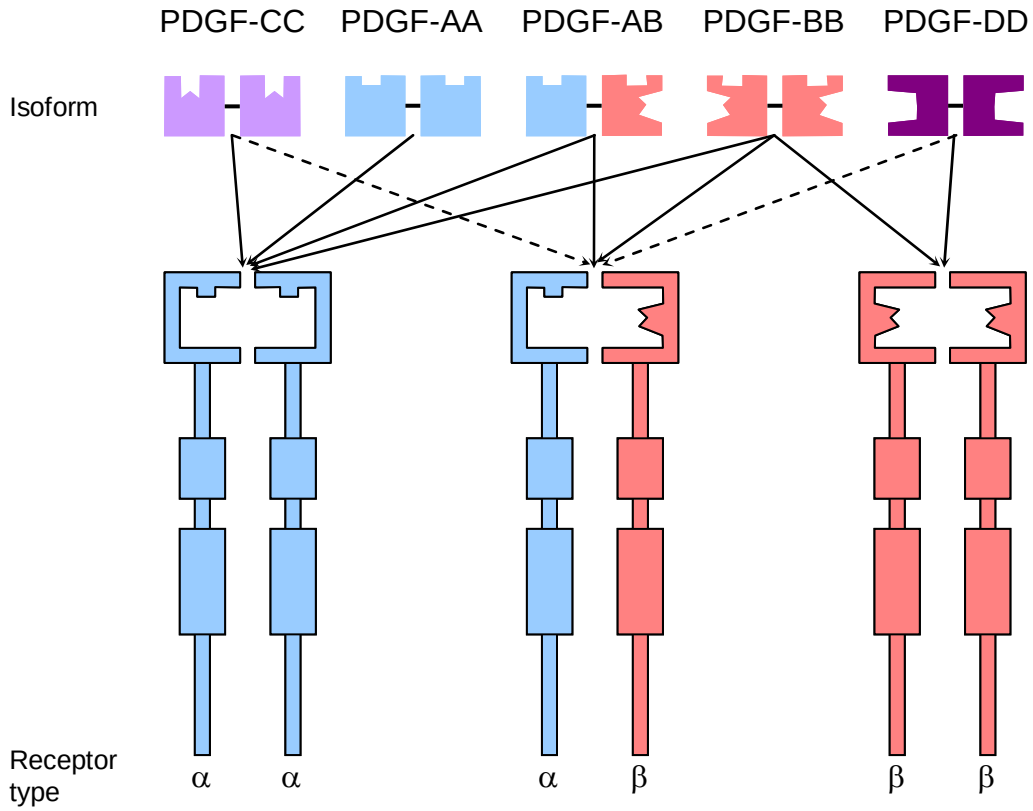


Figure 2. Receptor binding specificity of the five PDGF isoforms. The ability of the five different dimeric isoforms of PDGF to bind and activate homo- and heterodimeric complexes of PDGFRα and PDGFRβ are indicated by solid arrows. The dashed arrows indicate that receptor heterodimers can be activated. The figure is adopted from Li *et al*. (Li and Eriksson, 2003)

10.3 PDGF in physiology and pathophysiology

PDGF fulfills important functions during embryogenesis, in particular for the development of the kidneys, the lungs, the CNS and blood vessels (Leveen *et al.*, 1994; Soriano, 1994; Yeh *et al.*, 1991; Sasahara *et al.*, 1991). In these organs, connective tissue-like cell types are dependent on PDGF. The significant role of PDGF in the formation of connective tissue is also important during wound healing in the adult.

Moreover, aberrant synthesis of PDGF and concomitant autocrine growth stimulation are an important step in the development of diseases such as atherosclerosis or cancer. Animal models established a role for PDGF signaling in cardiovascular disease. For example, balloon injury of a normal vessel is a well accepted model to examine the role of different cell types and growth factors. mRNA levels of PDGF and its receptors are increased in the rat carotid artery after percutaneous transluminal coronary angioplasty (PTCA) and most luminal VSMCs are strongly positive for PDGF and its receptors (Majesky *et al.*, 1990). Further experiments investigated the actions of PDGF *in vivo* by infusing PDGF-BB into the rat carotid artery after a gentle, filament-mediated endothelial denudation without injuring the underlying medial VSMCs. The resulting data support a role for stimulation of migration, proliferation and altered gene expression of VSMCs by PDGF-BB (Jawien *et al.*, 1992; Bendeck *et al.*, 1996).

11 Phosphatidylinositol 3-kinases

11.1 Phosphatidylinositols (PtdIns)

PtdIns are composed of glycerol, two fatty acids and inositol 1-phosphate. Glycerol builds the backbone. In physiologic conditions, stearic acid is attached at position 1 while at position 2 arachidonic acid is attached. Inositol 1-phosphate is bound to position 3 (Payrastre *et al.*, 2001). The inositol residue can be phosphorylated additionally at positions 3, 4 and 5. These phosphorylated derivates are referred to as phosphoinositides (PtdInsPs) (Payrastre *et al.*, 2001; Vanhaesebroeck and Alessi, 2000). By further processing, these phospholipids can take on different functions. Phospholipases can cleave the inositol thus generating inositol tri-phosphate (IP_3) which serves as second messenger. Interaction with intracellular proteins or changes in the topology of membranes are two other possibilities (Payrastre *et al.*, 2001).

11.2 Phosphatidylinositol 3-kinases – classification, activation and inhibition

There are three different kinds of kinases capable of phosphorylating phosphatidylinositol or phosphoinositides: phosphoinositide 3-kinases (PI3Ks), phosphoinositide 5-kinases and phosphoinositidephosphate 4-kinases. PI3Ks are responsible for the production of PtdIns(3)P, PtdIns(3,5)P_2, PtdIns(3,4)P_2 and phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3). They are not substrate of any known PLC and function as second messengers by interacting directly with functional protein domains, such as pleckstrin homology domains (PH). Class I PI3Ks are the only enzymes that use PtdIns(4,5)P_2 as a substrate to synthesize PtdIns(3,4,5)P_3 (Rameh and Cantley, 1999). The latter is of special interest as it is a prerequisite to activate the serine/threonine kinase Akt, the primary mediator of PI3K-initiated signaling (see also chapter 12) (Payrastre *et al.*, 2001; Cantley, 2002).

Quiescent cells only contain significant levels of PtdIns(3)P, whereas other 3-phosphorylated phosphoinositides can hardly be found. After cellular stimulation, however, there is a drastic increase in 3-phosphorylated phosphoinositides (Vanhaesebroeck *et al.*, 2001). Class I PI3Ks seem to phosphorylate primarily PtdIns(4,5)P_2, *in vivo*, thereby generating PtdIns(3,4,5)P_3. The termination of PI3K signaling by degradation of PtdIns(3,4,5)P_3 can by mediated by phosphatases such as the PTEN tumor suppressor protein which can dephosphorylate PtdIns(3,4,5)P_3 at the 3-position and the Src homology-2 (SH2)-containing phosphatases, which dephosphorylate at the 5-position (Cantley, 2002).

Class I PI3Ks can be divided into two subclasses, class IA and class IB. Class IA PI3Ks are composed as heterodimers of the following: an inhibitory adaptor/regulatory (p55 / p85) subunit which p85 as prototype and a catalytic (p110) subunit. p85 exists in two subclasses (p85α/p85β) and contains a Src homology-3 (SH3) domain, a breakpoint cluster-region, two proline rich regions as well as two carboxy-terminal SH2 domains separated by an inter-SH2 region. This region mediates the binding of p85 to the catalytic subunit p110

(Wymann and Pirola, 1998). The SH2 domain of p85 exerts two functional activities. First, it binds to phospho-tyrosines being displayed on receptor tyrosine kinases after growth factor stimulation. Thereby, PI3K is targeted to the membrane leading to an increased activity of PI3K. The second function consists in p85 binding and integrating signals from various cellular proteins, including intracellular proteins such as PKC, Shp-2, Rac, Rho, Ras and Src, providing an integration point for activation of p110 and downstream molecules (Hennessy *et al.*, 2005).

There are three known isoforms of class IA p110 (p110α, p110β, p110δ), which contain an amino-terminal p85/p55-interacting region, a domain that binds to Ras, a phosphoinositide 3-kinase family accessory domain (PIK domain), which function is not fully clear yet but it is believed to be involved in substrate presentation (Flanagan *et al.*, 1993), and a carboxy-terminal catalytic domain.

Class IB PI3Ks consist of p110γ and a regulatory subunit p101. They are activated directly by G-protein coupled receptors and indirectly by other receptors. Of note, all class I PI3Ks also possess intrinsic serine/threonine protein kinase activity (Hennessy *et al.*, 2005).

Two chemical, cell permeable inhibitors have been used to inhibit the function of PI3K: wortmannin, a fungal metabolite that irreversibly inhibits p110 by covalent modification of the catalytic subunit (Wymann *et al.*, 1996), and the flavonoid derivative LY294002 a reversible, competitive inhibitor of the ATP binding site. However both inhibitors exhibit off-target activity, when used in higher doses than required for PI3K inhibition (Fruman *et al.*, 1998).

12 Akt and mitogen-activated protein kinases (MAPKs)

12.1 Protein kinase B (PKB) / Akt

Akt was initially identified as a homologue of the oncogene v-Akt, encoded by the genome of the murine lymphoma virus AKT8. Due to sequence homologies with protein kinase A (PKA) and protein kinase C (PKC), Akt is also referred to as protein kinase B (PKB) (Vogt *et al.*, 2006). It is a member of the AGC

(protein kinase **A** / protein kinase **G** / protein kinase **C**-like) class of serine/threonine kinases. Like the other members of the AGC-family, Akt requires phosphorylation to be activated. Except a spliced isoform of PKBγ/Akt3 all members need to be phosphorylated at two residues: a threonine (residue 308 in PKBα/Akt1) within the kinase t-loop and a serine in the hydrophobic motif (serine 473 in PKBα/Akt1). The activation of Akt depends on the generation of 3-phosphorylated phosphoinositides generated by class I PI3-kinases. These lipids interact with the PH domain present in proteins including Akt and phosphoinositide-dependend kinase 1 (PDK1), thereby recruiting these proteins to the membrane (Scheid and Woodgett, 2001).

This relocation from the cytoplasm to the inner surface of the plasma membrane places Akt, the primary mediator of PI3K-initiated signaling, in close proximity to the regulatory kinases responsible for its phosphorylation and activation. Upon binding to PIP3, Akt undergoes conformation changes that allow the phosphorylation by PDK1 at Thr^{308} in the activation loop. Subsequent phosphorylation at Ser^{473} by a still unidentified kinase, referred to as PDK2, results in full Akt activation (figure 3). Possible PDK2 candidates are integrin-linked kinase, Akt itself, PDK1, DNA-dependent protein kinase, mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK-2) or the TOR/Rictor complex (Nicholson and Anderson, 2002; Vogt *et al.*, 2006; Taniyama *et al.*, 2004).

The serine/threonine phosphatases PP2A is responsible for the inactivation of Akt (Millward *et al.*, 1999).

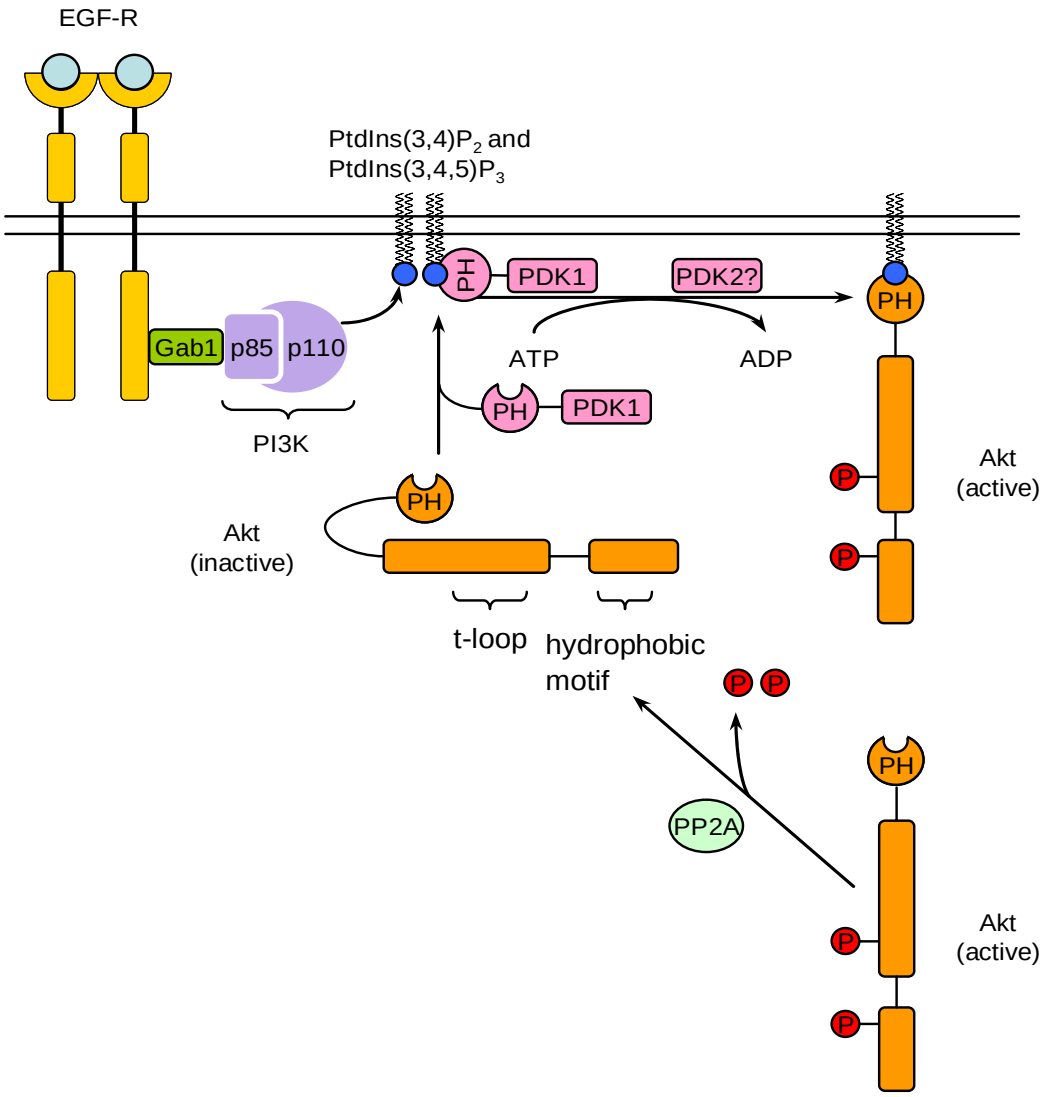


Figure 3. Activation of PKB/Akt downstream of EGF receptor. Recruitment of PI3K to the activated EGF-R *via* the adaptor molecule Gab1 generates 3-phosporylated phosphoinositides at the membrane which serve as an anchor for PH domain containing proteins. At the membrane Akt is phosphorylated by PDK1 and a yet unidentified kinase (termed PDK2). Active Akt translocates to the cytosol and the nucleus. Inactivation occurs *via* protein phosphatase 2A. The figure is adopted from Vanhaesebroeck and Alessi, 2000 (Vanhaesebroeck and Alessi, 2000).

Among the first identified Akt substrates was glycogen synthase kinase 3 (GSK3). This protein is inactivated upon phosphorylation. Akt triggers a signaling network that positively regulates G1/S cell cycle progression through inactivation of GSK3 (Liang and Slingerland, 2003). By phosphorylating GSK3, Akt stabilizes or even activates GSK3 targets such as cyclin D, c-Myc and

glycogen synthase, enzymes involved in the progression of the cell cycle (Cohen and Frame, 2001).

Further Akt targets belong to the FOXO family of transcription factors. These factors enhance e.g. transcription of the cell cycle inhibitor $p27^{Kip1}$. Phosphorylation of FOXO transcription factor results in inactivation *via* cytoplasmic retention or inhibition of DNA binding (Burgering and Kops, 2002; Scheid and Woodgett, 2003; Liang and Slingerland, 2003).

Akt is also referred to as survival kinase in that it is able to inactivate the pro-apoptotic proteins BAD and caspase 9 by phosphorylation, which leads to cellular survival.

Akt supports protein synthesis, as it occurs in hypertrophy. It does so *via* phosphorylation and activation of mammalian target of rapamycin (mTOR). This effect is promoted by the GSK3-dependent activation of eukaryotic initiation factor 2B (eIF2B) (Cohen and Frame, 2001; Scott *et al.*, 1998).

The Akt pathway includes a cross-talk between Akt and Erk1/2. This cross-talk eventually leads to inactivation of the Erk1/2 pathway (Galetic *et al.*, 2003; Reusch *et al.*, 2001).

12.2 MAPKs

Mitogen-activated protein kinases (MAPKs) are a highly conserved, ubiquitously expressed family of serine/threonine kinases which control a great variety of cellular processes, such as transformation, differentiation, apoptosis and cell growth. These kinases require a dual phosphorylation to be activated. Specific phosphatases are responsible for the inactivation of MAPKs. This interplay of phosphorylation and dephosphorylation enables the cell to rapidly adjust its requirement of active or inactive MAPKs (Johnson and Lapadat, 2002).

MAPKs are activated and regulated by a signaling cascade comprised of three successively activated kinases. MAPKs are activated by highly selective MAPK kinases (MKK or MEK), which are in turn activated by MAPK kinase kinases (MKKK or MEKK) (figure 4). Major targets of the MAPKs are other protein

kinases, phospholipases and last but not least transcription factors (Johnson and Lapadat, 2002).

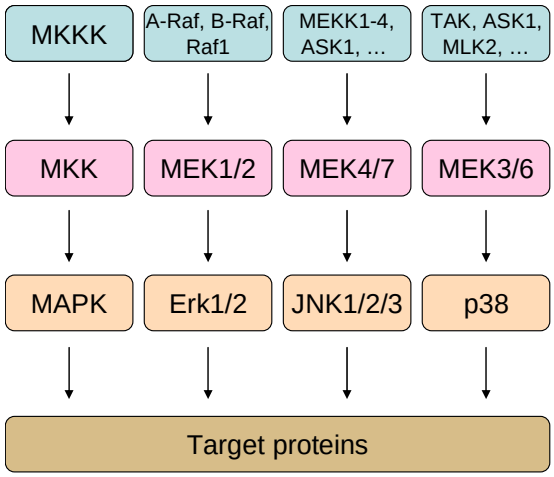


Figure 4. The MAPK cascade. The modules shown are representative of pathway connections for the respective MAPK. The left column represents the general cascade, whereas the second to fourth column show the three subfamilies of Erk, Jnk and p38. There are multiple component MKKKs, MKKs, and MAPKs for each system. Mitogens and cellular stresses lead to activation of these cascades.

To date, several distinct groups of MAPKs have been characterized in mammals: extracellular signal-regulated kinases (Erks) 1 and 2 (Erk1/2), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 isoforms α, β, γ, and δ, Erk5/BMK1 and the orphan MAPKs Erks 3, 7, nemo-like kinase (NLK) and MOK (Chen *et al.*, 2001; Kondoh *et al.*, 2005; Kyriakis and Avruch, 2001).

Three of these subfamilies of the MAPKs are of interest for this study. The extracellular-signal regulated kinases (Erk1/2) are critically involved in proliferation upon mitogenic stimulation. Targets are cytoplasmic proteins, membrane proteins, cytoskeletal proteins, and nuclear proteins including transcription factors (Chen *et al.*, 2001). The c-Jun N-terminal kinases (JNKs), also referred to as stress activated protein kinases (SAPK), activate the transcription factor c-Jun, a constituent of the transcription factor AP-1 (Takahashi and Berk, 1998). The p38 MAPK isoforms regulate the production of key inflammatory mediators, including TNF-α, IL-1β , and COX-2. Further it has been shown to be crucially involved in the regulation of the cell cycle

(Ambrosino and Nebreda, 2001; Bulavin and Fornace, Jr., 2004; Schieven, 2005).

13 Caveolins

The discovery of caveolae is accredited to the Nobel laureate George Emil Palade (Palade, 1953), who called them *plasmalemmal vesicles*. Two years later, E. Yamada described similar structures in the gall bladder epithelium, naming them *caveolae intracellulares* due to their appearance (YAMADA, 1955). Caveolae are flask-shaped vesicular invaginations of the plasma membrane 50–100 nm in diameter. The main structural proteins of caveolae are the caveolins. The latter form a scaffold onto which many classes of signaling molecules can assemble to generate preassembled signaling complexes. In addition to concentrating these signal transducers within a distinct region of the plasma membrane, caveolin binding may functionally regulate the activation state of caveolae-associated signaling molecules. In recent years interest in caveolae increased more and more due to improved insight in caveolin function and caveolae-associated signal transduction (Williams and Lisanti, 2004b). Caveolin proteins are expressed in most of the cell types that play a role in the development of atherosclerosis. These cells include endothelial cells, macrophages and smooth muscle cells (Frank and Lisanti, 2004). The next paragraphs will show recent findings essential for this study.

13.1 Discovery and structure

The caveolin gene family has three members in vertebrates: caveolin-1, caveolin-2, and caveolin-3. The caveolins are integral membrane proteins of 18-24 kDa. Structurally, caveolin can be roughly divided into three distinct domains: the hydrophilic amino-terminal, the hydrophobic central stretch and the hydrophilic carboxy-terminal. They assume an unusual hairpin-like structure within the membrane, with both amino and carboxy terminus facing the cytoplasm (Schlegel *et al.*, 1998) (figure 5 A+B). This atypical membrane-spanning model is supported by findings that antibodies against the amino or

carboxy terminus of caveolin-1 need permeabilized cells in order to bind caveolin-1. Moreover cell-surface biotinylation is not capable of labeling caveolin-1. Finally, palmitoylation and tyrosine phosphorylation of both the amino and carboxy-terminal domains provide further evidence of this assumption since both reactions are post-translational modifications exclusively located in the cytoplasm (Williams and Lisanti, 2004a). Caveolin-1 (also known as caveolin, Cav-1, or VIP21) is ubiquitously expressed with highest levels in adipocytes, endothelial and smooth muscle cells. It was the first member of the caveolin family to be described. It was shown to be a structural constituent of caveolae (Rothberg *et al.*, 1992) and was isolated as one of several proteins that became phosphorylated on tyrosine residues in chicken embryo fibroblasts transformed with the v-Src oncogene (Glenney, Jr. and Zokas, 1989). Cloning of the Cav-1 cDNA revealed that it was identical to another protein, VIP21, which had been cloned almost simultaneously (Glenney, Jr. and Soppet, 1992; Kurzchalia *et al.*, 1992). Interestingly, VIP21 was isolated as an integral membrane protein component of transport vesicles derived from the trans-Golgi network in Madin-Darby canine kidney (MDCK) cells, suggesting that Cav-1/VIP21 may have a role in molecular trafficking as well as oncogenesis. Further studies revealed that caveolin-1 can be subdivided in two isoforms (α and β) from which the β-isoform was originally thought to arise from an internal translational start site but it has recently been shown to be translated from different mRNA than caveolin-1α (Krajewska and Maslowska, 2004). Caveolin-1β is 31 amino acids shorter compared to the α-isoform.

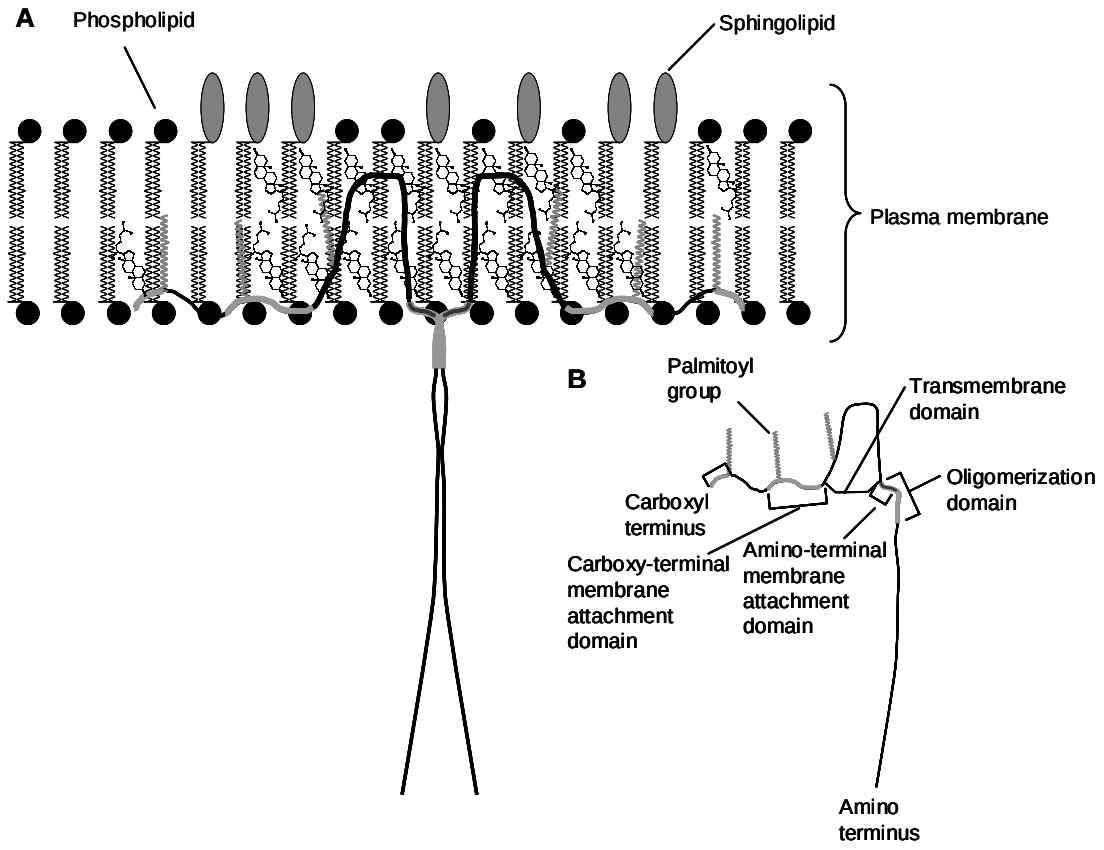


Figure 5 Primary structure and topology of caveolin-1. A, the current view of caveolin-1 localization in the plasma membrane. Two caveolin-1 monomers forming a dimmer are shown for simplicity. Usually 14-16 monomers self-associate to form a single caveolin homo-oligomer or hetero-oligomer with caveolin-2 (not shown). Modified from Razani *et al.* (Razani *et al.*, 2002b). **B**, caveolin-1 domains. Note that the amino-terminal membrane-attachment domain is also referred to as the caveolin scaffolding domain (CSD).

Caveolin-2 and caveolin-3 were both identified in 1996. Caveolin-2 was discovered by microsequencing of a 20kDa protein that co-purified with adipocyte-derived caveolar membranes (Scherer *et al.*, 1996). Caveolin-2, similarly to caveolin-1, has been found to exist in multiple isoforms that differ in the length of their amino-terminal part. Without caveolin-1, caveolin-2 exists mainly as monomer or homodimer that is retained at the level of the Golgi complex (Krajewska and Maslowska, 2004; Manninen *et al.*, 2005).

Caveolin-3 (also known as M-caveolin; a muscle specific member of the caveolin family) was identified through database search and traditional cDNA library screening in attempt to find caveolin-1 homologues (Way and Parton,

1995; Tang *et al.*, 1996). It is closely related to caveolin-1 as shown by protein sequence homology. It is able to form stable homooligomers and its expression is sufficient to generate caveolae (Krajewska and Maslowska, 2004).

13.2 Localization and functions in pathology

Some groups consider caveolae to be a subset of lipid rafts, highly ordered microdomains within the plasma membrane. However, since some proteins are known to localize selectively to either lipid rafts or caveolae but not both this assumption may not be completely accurate (Williams and Lisanti, 2004a). One evidence for the thesis that lipid rafts and caveolae are separate entities is that lipid rafts do not contain caveolins and caveolae lack glycosyl-phosphatidylinositol-anchored proteins (Li *et al.*, 2005). Current research is establishing the importance of caveolins in modulating the development and progression of a variety of diseases. The following paragraphs will show where the caveolins are expressed and will give a brief synopsis of the involvement of caveolins in various pathological disorders, especially related to the cardiovascular system.

Since caveolin-3 has been identified as the main caveolar protein expressed in skeletal and cardiac myocytes, extensive research has elucidated its imolication in skeletal myopathies such as rippling muscle disease (RMD), distal myopathy (DM), and idiopathic hyperCKemia (Betz *et al.*, 2001; Kubisch *et al.*, 2003; Carbone *et al.*, 2000; Woodman *et al.*, 2004).

Previous studies showed that caveolin-2 is coexpressed with caveolin-1 in most cell types and colocalizes with caveolin-1 in caveolae, forms hetero-oligomers with caveolin-1, and requires caveolin-1 for proper membrane localization. It has been proposed that caveolin-2 may function as an accessory protein of caveolin-1 (Krajewska and Maslowska, 2004). However, caveolin-2 deficient mice show evidence of severe pulmonary dysfunction without disruption of caveolae indicating a selective role of caveolin-2 in mammalian physiology independent of caveolin-1 (Razani *et al.*, 2002a).

Caveolin-1 is most abundantly expressed in terminally differentiated cells such as epithelial and endothelial cells, adipocytes, fibroblasts, and smooth muscle cells (Glenney, Jr., 1992; Kurzchalia *et al.*, 1992; Kurzchalia *et al.*, 1994; Rothberg *et al.*, 1992). Depending on cell type caveolin-1 seems to exist either as soluble cytoplasmic form or as secreted form (Liu *et al.*, 2002; Krajewska and Maslowska, 2004) and the 31 amino acids lacking in the β-isoform seem to play a role in selectively targeting the isoforms to different cellular compartments (Williams and Lisanti, 2004a).

As mentioned above, post-translational modifications take place in both the amino-terminal and the carboxy-terminal domains. The C-terminus is palmitoylated on three cysteine residues at positions 133, 143, 156, while the N-terminus is phosphorylated at tyrosine 14 (Stan, 2005). The function of the palmitate chains seems to be complex. They possibly anchor the carboxy-terminal region to the membrane and they may increase the stability of the oligomers and therefore the scaffold structure of caveolae as well (Krajewska and Maslowska, 2004). Caveolin-1 selectively binds cholesterol and affects cholesterol homeostasis and it has been suggested that at least two palmitate chains are required for the binding and transport of cholesterol (Uittenbogaard and Smart, 2000; Fielding and Fielding, 2001).

The tyrosine phosphorylation is essential for protein-protein interaction. Mutation of the tyrosine 14 residue that prevents phosphorylation was shown to impair association with the Grb7 protein involved in signal transduction (Lee *et al.*, 2000).

Involvement in cellular transformation was the first feature of caveolin-1 to be suggested as it was identified as a substrate for v-Src in transformed chicken embryo fibroblasts (Glenney, Jr., 1989). Further studies revealed that caveolin-1 levels in oncogenically transformed cells are often down regulated and increased growth correlates with a loss of caveolin-1 expression (Koleske *et al.*, 1995). Cardiovascular disorders such as vascular dysfunction, atherosclerosis and neointimal hyperplasia strongly correlate with caveolin-1 deficiency. Caveolin-1 null mice display vascular dysfunction, with defects in endothelium-

dependent contraction, mediated mainly through the loss of inhibition of eNOS activity (Razani *et al.*, 2001; Drab *et al.*, 2001). The potent eNOS inhibiting activity of caveolin-1 was demonstrated using cell permeable proteins linked to the CSD (Bucci *et al.*, 2000). Vascular permeability is another feature to be linked to caveolin-1 function. Endothelial cells highly express caveolin-1 and the latter is clearly linked to transcytotic processes, as aortic ring segments from caveolin-1 null mice do not efficiently take up macromolecules, such as albumin (Schubert *et al.*, 2001). The importance of caveolin-1 in maintaining vascular tone, permeability, and normal physiology suggests that caveolin-1 may have a role in pathogenic vascular conditions such as atherosclerosis. Further evidence for this assumption was provided by a recent study where neointimal hyperplasia was significantly enhanced in the carotid arteries of caveolin-1 null mice (Hassan *et al.*, 2004).

C MATERIALS AND METHODS

1 Stock solutions

1.1 Preparation of Angiotensin II (Ang II) solution

Ang II (Sigma) was dissolved in 0.25 % sterile BSA solution (Roche) and stored at -80°C as a 2 mM stock solution. For working aliquots the stock solution was diluted to 20 µM with 0.25 % BSA and stored at -20°C. 100 nM Ang II were used for experiments.

1.2 Preparation of EGF solution

Following a quick centrifugation of the vial containing the EGF lyophilisate (Upstate), a 100 µg/ml stock solution (EGF) was prepared in sterile phosphate buffered saline (PBS; see also paragrapgh 2.2) and stored at -20°C. 100 ng/ml EGF was used for experiments.

1.3 Preparation of PDGF-BB solution

After a quick centrifugation, lyophilized, rec. PDGF-BB (Bachem) was reconstituted in 10 mM acetic acid to a concentration of 100 µg/ml. This solution was then diluted with sterile PBS + 0.1 % BSA to a concentration of 10 µg/ml and stored at -20°C. 20 ng/ml PDGF was used for experiments.

1.4 Preparation of Resveratrol (RV)

trans-resveratrol (Sigma) was dissolved in DMSO to concentrations of 50 or 100 mM respectively and stored at -20°C.

1.5 Preparation of caffeic acid phenethyl ester (CAPE) solution

5 mM stock solutions of CAPE (Calbiochem) were prepared in DMSO and stored in 15µl aliquots at -20°C.

2 Cell Culture

2.1 Bovine serum

Bovine serum (Invitrogen) was heat inactivated for 30 minutes at 56°C and stored at -20°C.

2.2 Solutions

PBS

NaCl		7.20 g
Na_2HPO_4		1.48 g
KH_2PO_4		0.43 g
H_2O	ad	1000.0 ml

PBS$^{\oplus}$

NaCl		8.00 g
KCl		0.20 g
Na_2HPO_4		1.15 g
KH_2PO_4		0.20 g
$MgCl_2$ x 6 H_2O		0.10 g
$CaCl_2$ x 2 H_2O		0.10 g
H_2O	ad	1000.0 ml

TBS-T

Tris-base		3.0 g
NaCl		11.1 g
Tween-20		2.0 ml
H_2O	ad	1000.0 ml

Trypsin/EDTA (T/E)

Trypsin (diluted 1+249 in PBS)		0.05 g
EDTA		0.20 g
PBS	ad	100.0 ml

Digestion buffer

Collagenase (272 U/mg) (Biochrom)		0.100 g
HEPES		0.240 g
Ascorbic acid		0.005 g
BSA		0.100 g
HAM's F12 medium (Pan Biotech)	ad	100.0 ml

Freezing medium

DMEM	8.0 ml
DMSO	1.1 ml
Bovine serum	2.0 ml

2.3 Cells

For all experiments with CAPE VSMCs from male Sprague-Dawley rat (Charles River Wiga) thoracic aortas were used unless stated otherwise.

3T3 immortalized fibroblasts from Shp-2 exon $3^{-/-}$ (Shp-$2^{\Delta 46\text{-}110}$) mice were described previously ((Saxton *et al.*, 1997); named Shp-$2^{-/-}$). Shp-$2^{-/-}$ cells which contain a stably reconstituted Shp-2 protein *via* retroviral transfection (Zhang *et al.*, 2002) were used as control (Shp-2 rescue 3T3 fibroblasts). All Shp-2 mutants were a kind gift of Prof. Benjamin G. Neel, Cancer Biology Program, Beth Israel Deaconess Medical Center, Boston, MA 02115, USA. The fibroblasts were cultured as described for VSMCs.

2.4 Isolation of VSMCs

VSMCs were isolated by enzymatic digestion. Aortas were removed and placed in PBS$^{\oplus}$ for further preparation. Afterwards, the aortas were opened up and placed in digestion buffer (on the basis of (Palmberg *et al.*, 1985)) for 15 minutes at 37°C. Then the endothelium and adventitia were removed with tweezers before mincing the aortas with two scalpells. Subsequently the vessels were incubated in digestion buffer for another 4 hours at 37°C. After centrifugation at 220g for 10 minutes, cells were placed in a 25 cm² flask and grown in DMEM w/o phenol red (BioWhittaker) supplemented with 10 % bovine serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (growth medium). At least four aortas are needed for one 25 cm² flask. VSMCs were identified by fluorescence microscopy using a monoclonal anti-α-smooth muscle actin FITC-conjugated antibody (1:250 dilution Sigma) (Chamley-Campbell *et al.*, 1979). Human umbilical vein endothelial cells (HUVECs), Raw 264.7 and L929 murine fibroblasts were used as negative controls to test the specificity of the antibody. However, for most experiments, VSMCs kindly provided by Prof. Kathy K. Griendling, Emory University, Atlanta, isolated by the same method, were used.

2.5 Freezing, storage and thawing of VSMCs

For freezing, VSMCs from one 75 cm² flask were trypsinized, counted, spun down and resuspended in ice-cold freezing medium to a concentration of $1 \cdot 10^6$ cells/ml. 1 ml aliquots were frozen in cryovials at -20°C for 24 hours, then at -80°C for another 24 hours before long time storage at -196°C in liquid nitrogen. For thawing, cells were warmed to 37°C and immediately dissolved in pre-warmed culture medium. After centrifugation, cells were resuspended in culture medium and placed in a 75 cm² flask.

2.6 Passaging of cells

Cells were passaged twice a week. Growth medium was removed and cells were washed once with 10 ml PBS before incubation with trypsin/EDTA solution for approximately 2 minutes to allow detachment of the cells. Cells were disseminated with $1 \cdot 10^6$ or $1.9 \cdot 10^6$ cells respectively in 75 cm² culture flasks. For experiments VSMCs were disseminated in appropriate plates. Cells were cultured between passage 7 and 15 in 6- or 24-well plates or 60 mm dishes and used at 80-90 % confluence.

When Ang II or EGF was used for stimulation, cells were serum-starved in DMEM containing 0.1 % bovine serum overnight prior to experiments. For serum-stimulation or stimulation with PDGF cells were rendered quiescent by cultivation in DMEM without serum for at least 24 hours.

3 Cell counting

3.1 Experimental procedure

Cells were seeded at a density of 100,000 cells/well in six-well plates and kept in growth medium for 24 h. After serum starvation for 4 days, cells were pre-treated with or without 5 µM CAPE for 30 min and subsequently stimulated with 20 ng/ml PDGF. After serum starvation for 24 hours, cells were pre-incubated with vehicle, 5 µM SB203580 or CAPE at the indicated concentrations for

30 min and subsequently stimulated with 20 ng/ml PDGF for 48 hours. Cells were then trypsinized and the cell number was determined in a trypan blue exclusion assay using a Vi-Cell cell counter (Beckman, Krefeld, Germany).

4 Western blot analysis

4.1 Solutions

Lysis buffer “stock concentrate”

HEPES	50 mM
NaCl	50 mM
EDTA	5 mM
$Na_4P_2O_7$ x 10 H_2O	10 mM
NaF	50 mM
Na_2VO_4	1 mM

Bring volume up to 325 ml with H_2O. Adjust pH with NaOH at 4°C to pH 7.5. Replenish with H_2O to a final volume of 430 ml. Store at 4°C.

Prepare 1x lysis buffer just before use as follows.

Stock concentrate	8.6 ml
PMSF	1.0 mM
Complete® 25x (Roche)	0.4 ml
Triton X-100 (10 % in H_2O)	1.0 ml

SDS sample buffer (stock solution w/o β-mercaptoethanol)

0.5 M TRIS-HCl (pH 6.8)	37.5 ml
SDS	6.0 g
Glycerol	30.0 ml
Bromophenol blue	15.0 mg
H_2O ad	100.0 ml

SDS sample buffer (3x)

Stock solution	850 µl
β-mercaptoethanol	150 µl

To prepare 1x solution, add the appropriate amount of water to the 3x solution.

Resolving gel 10 %

Rotiphorese® Gel 30 (Roth)	5.00 ml
TRIS-base 1.5 M, pH 8.8	3.75 ml
SDS 10 %	0.15 ml
H_2O	6.10 ml
TEMED	15 µl
APS	75 µl

Stacking gel

Rotiphorese® Gel 30 (Roth)	1.7 ml
TRIS-HCl 1.25 M, pH 6.8	1.0 ml
SDS 10 %	0.1 ml
H_2O	7.0 ml
TEMED	20 µl
APS	100 µl

Gels were prepared using Rotiphorese® Gel 30 containing 30 % polyacrylamide (PAA)/0.8 % bisacrylamide. Depending on the molecular weight of the protein of interest, different concentrations of PAA were used. Before addition of TEMED and APS, the solutions were degassed using ultrasound for 10 minutes.

Electrophoresis buffer (5x)

TRIS-base		15.0 g
Glycine		72.0 g
SDS		5.0 g
H_2O	ad	1000.0 ml

To prepare 1x solution, add the appropriate amount of H_2O to the 5x solution

Blotting buffer (5x)

TRIS-base		15.2 g
Glycine		72.9 g
H_2O	ad	1000.0 ml

Blotting buffer (1x)

Blotting buffer (5x)	200 ml
Methanol	200 ml
H_2O	600 ml

Tris-buffered saline pH 8.0 containing 1 % Tween (TBS-T)

Tris-base		3.0 g
NaCl		11.1 g
Tween-20		1.0 ml
H_2O	ad	1000.0 ml

Coomassie staining solution

Coomassie brilliant blue G		1.5 g
Acetic acid (100 %)		50.0 ml
Ethanol (96 %)		225.0 ml
H_2O	ad	500.0 ml

Coomassie destaining solution

Acetic acid (100 %)		100 ml
Ethanol (96 %)		335 ml
H_2O	ad	1000 ml

Ponceau stock solution

Ponceau S		2.0 g
Acetic acid (100%)		50.0 ml
H_2O	ad	100.0 ml

Dilute the stock solution 1+9 with H_2O before usage

4.2 Principle

Separation of proteins was performed by denaturing discontinuous SDS-polyacrylamide gel electrophoresis (SDS-Disc-PAGE) (Laemmli, 1970). Using discontinuous electrophoresis, aggregation of proteins entering the gel is prevented and sharper bands are obtained. The gel matrix is divided in two areas, the narrow porous resolving gel and the wide porous stacking gel. Furthermore two different buffers are used. The buffer for the resolving gel contains 0.375 mol/l Tris-HCl at pH 8.8, the buffer for the stacking gel contains 0.125 mol/l Tris-HCl at pH 6.8. The latter pH is near the isoelectric point of glycine in the electrophoresis buffer. Thus at the beginning of the electrophoresis glycine possesses a low electrophoretic mobility whereas chloride anions possess a very high mobility. The proteins to be separated exhibit mobilities between those of glycine and chloride. When applying an electric field, in this discontinuous system all ions are moving with the same speed, building a stack in order of their mobilities. This phenomenon is called

isotachophoresis. As soon as the stack reaches the narrow porous resolving gel, the proteins receive a frictional resistance and the zone gets sharpened. Glycine is not affected and overtakes the proteins thus the system gets homogenous and the proteins will now be separated by their size.

4.3 Experimental procedure

4.3.1 Sample preparation

Cells grown in 60 mm dishes were stimulated with the indicated substances for the denounced times. RV, CAPE and inhibitors (table 1) were preincubated for 30 minutes respectively.

Cells were harvested on ice by washing twice with ice-cold PBS and addition of 200 µl lysis buffer. After 30 minutes incubating on ice, cells were scraped off and lysates were transferred to pre-cooled Eppendorf Tubes®. The homogenates were cleared by centrifugation at 14,000 g for 10 minutes at 4°C. Protein concentration in the supernatants was determined by the BCA assay (figure 6) using a Tecan Sunrise™ absorbance reader. The remaining supernatants were aliquoted and mixed with 3x lysis buffer. Samples were boiled at 95°C for five minutes and stored at -20°C.

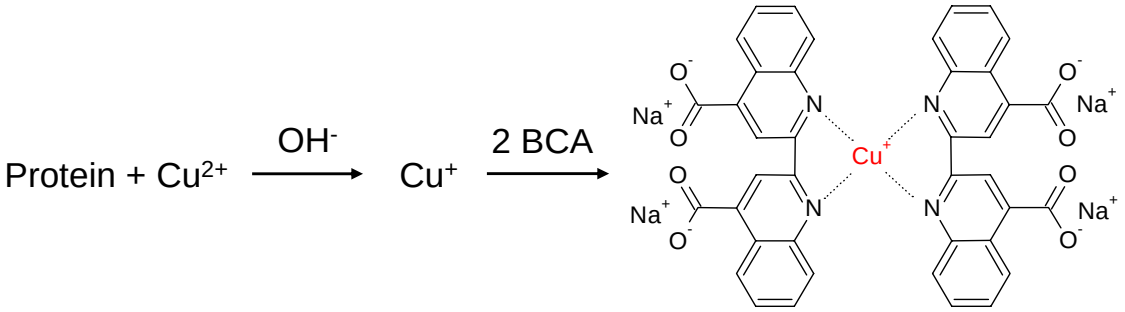


Figure 6: BCA assay

Table 1: inhibitors and stimulants

Name	Concentration	Provider
AG 1478	250 nM	Qbiogene-Alexis
Anisomycin	10µM	Sigma
Aphidicolin	1 µM	Calbiochem
CoPP	10 µM	Alexis
NAC	10 mM	Sigma
PD90580	20 µM	Qbiogene-Alexis
PP1	20 µM	Qbiogene-Alexis
SB203580	5-10 µM	Calbiochem
SnPP	10 µM	Alexis
Wortmannin	50 nM	Qbiogene-Alexis

4.3.2 Electrophoresis, tank blotting and protein detection

Equal amounts of protein were loaded and separated by SDS-Disc-PAGE (Mini Protean 3, Bio-Rad, Munich, Germany). Proteins were then transferred either for 60 min at 100 V or overnight at 25 V to a nitrocellulose membrane (Hybond™ ECL™, Amersham Biosciences) with a Mini Trans-Blot® Cell (Bio-Rad, Munich, Germany). After blocking with non-fat dry milk (Bio-Rad, Munich, Germany) in TBS-T for one hour, primary antibodies (table 2) dissolved in TBS-T containing 5 % BSA were added and rocked gently overnight at 4°C. Subsequently the membranes were washed three times with TBS-T and then incubated with appropriate horseradish-peroxidase conjugated secondary antibodies (table 3) for one hour at room temperature. Immunoreactive bands

were detected with an enhanced chemo luminescent detection kit (ECL Plus™, Amersham, Freiburg, Germany) and x-ray films (Fuji) The films were developed with an AGFA Crurix 60 (AGFA, Cologne, Germany) and scanned for digital analysis.

Table 2: primary antibodies

Target	Source	Dilution	Provider
Akt	rabbit	1:2000	Cell Signaling
Caveolin 1	mouse	1:1000	BD Biosciences
EGF-R	rabbit	1:1000	Cell Signaling
Erk 1/2 (p42/44)	rabbit	1:5000	Cell Signaling
HO-1 (HSP32)	rabbit	1:1000	Calbiochem
p70	rabbit	1:2000	Cell Signaling
phospho-Akt (ser^{473})	rabbit	1:2000	Cell Signaling
phospho-EGF-R (typ^{845})	rabbit	1:1000	Cell Signaling
phospho-Erk 1/2	rabbit	1:5000	Cell Signaling
phospho-p38	rabbit	1:1000	Cell Signaling
phospho-p70 (thr^{421}/ser^{424})	rabbit	1:2000	Cell Signaling
phospho-Tyr-100	mouse	1:2000	Cell Signaling
PI3-kinase p85	rabbit	1:4000	Upstate
retinoblastoma protein	mouse	1:1000	BD Biosciences

Table 3: secondary antibodies

Target	Source	Dilution	Provider
rabbit IgG HRP-conjugated	goat	1:10000	Jackson
mouse IgG1 HRP-conjugated	goat	1:1000	Serotec

Coomassie blue staining

In order to control equal loading and protein transfer, gels were stained with Coomassie blue staining solution after blotting. Gels were incubated in staining solution for 15 minutes and thereafter washed in destaining solution until protein bands were clearly visible.

Ponceau staining

Membranes were stained with Ponceau to ensure that proteins were evenly transferred. Briefly, membranes were incubated with Ponceau staining solution for 5 minutes and subsequently washed with demineralised water until protein bands were clearly visible. For removing the coloring the membrane was washed 4 times for 5 minutes with TBS-T.

5 Immunoprecipitation

5.1 Principle

Immunoprecipitation is used for the enrichment of certain proteins from cell lysates as well as for investigation of protein-protein interaction. Frequently, it is applied for the analysis of receptor tyrosine kinases and their scaffold or adaptor proteins, also referred to as co-immunoprecipitation. For that, antibodies specific for one protein of interest are added to the whole cell lysates. After formation of the antigen-antibody complex, this complex is precipitated with protein A, a bacterial protein *(*from *Staphylococcus aureus*) bound to agarose beads with high affinity to the F_c moiety of immunoglobulins. After purification of the immunoglobulin loaded beads, the immune complex is dissociated and the protein with its ligands is analyzed by Western blot.

5.2 Experimental procedure

Cells were treated and harvested as described under 4.2 unto protein determination. After the determination of the protein concentration, equal amounts (300 – 400 µg) of proteins were aliquoted and filled up to a final volume of 500 µl with lysis buffer. Afterwards the corresponding volume of the respective antibody was added to the lysates, according to the manufacturer's instruction and rocked gently overnight. For each sample, 50 µl of a 50 % solution of Protein A, immobilized on agarose beads, were centrifuged shortly, the supernatant was removed and the beads were resuspended in the adequate volume of lysis buffer. 50 µl of this suspension was added to each lysate und swayed gently for 3 hours. After this time, the bead solutions were centrifuged (10,000 x g, 5 minutes, 4°C), 40µl of the supernatant were kept as binding control and the beads were carefully washed three times with lysis buffer with a short run centrifugation after each washing step. Subsequently the wash solution was removed completely by centrifugation (10,000 x g, 5 minutes, 4°C), sample buffer containing β-mercaptoethanol was added to the pellet and the probe was heated to 95°C for 5 minutes to dissolve the protein from the

precipitate. After recentrifugation (10,000 x g, 30 seconds, 4° C), 30µl of each supernatant were analyzed *via* Western blot as described under 3.

6 Cell cycle analysis

6.1 Flow cytometry

6.1.1 Principle

Flow cytometry is a means of measuring certain physical and chemical characteristics of cells or particles as they travel in suspension one by one past a sensing point. To achieve a flow of cell one after another through a small capillary without blocking it, a combination of two physical phenomena is used.

1) Assuming a laminar flow of liquid through a tube, a viscous drag at the boundary will result in a higher velocity in the centre. The velocity profile resembles a parabola. The Bernoulli Effect associates these changes in speed with inverse alterations in pressure, such that any particle in the fluid will move toward and remain in the centre.

2) In order to prevent blocking, a suspension of cells is introduced through a wide test tube which is surrounded concentrically by a larger tube containing a sheath fluid. By constricting of this coaxial flow whilst maintaining a laminar flow a focused stream of cells is obtained (figure 7).

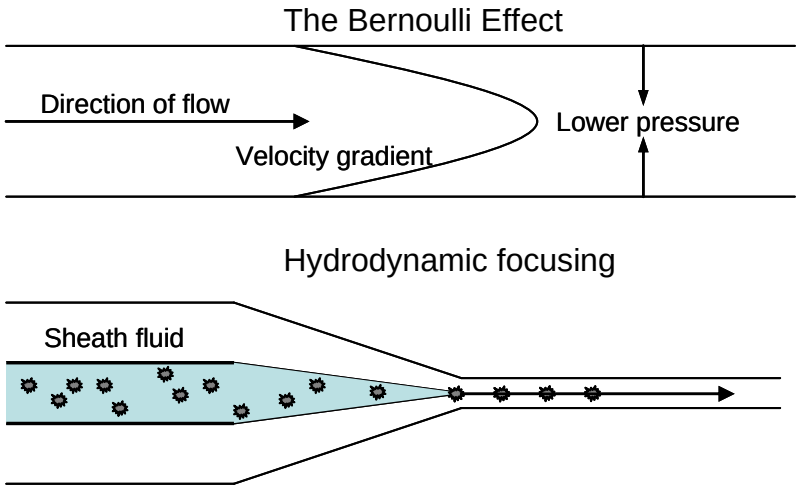


Figure 7: Fluidics and hydrodynamic focusing.

As stated above, flow cytometers are configured to examine several parameters of cells or particles simultaneously. To get these information the cells are exposed to a laser beam. As a cell passes through this laser beam several physical processes take place:

- absorption, diffraction, refraction and reflection of the incident light and
- emission of longer wavelengths following excitation and relaxation of electronic states (fluorescence)

Diffraction has an impact close to the optical axis and is closely related to the size of the object. This low angle scattered light represents an important parameter commonly referred to as “forward angle light scatter” (FSC). At higher angles, refraction and reflection become more and more important. These effects are caused by structural features of the cell, such as granularity and surface winding. These parameters are measured orthogonal to the stream and are referred to as “side scatter” (SSC). In adition fluorescence is also measured in an angle of 90° to the laser beam after separating various components by optical filtration. Figure 8 shows a typical arrangement for measurement of four components.

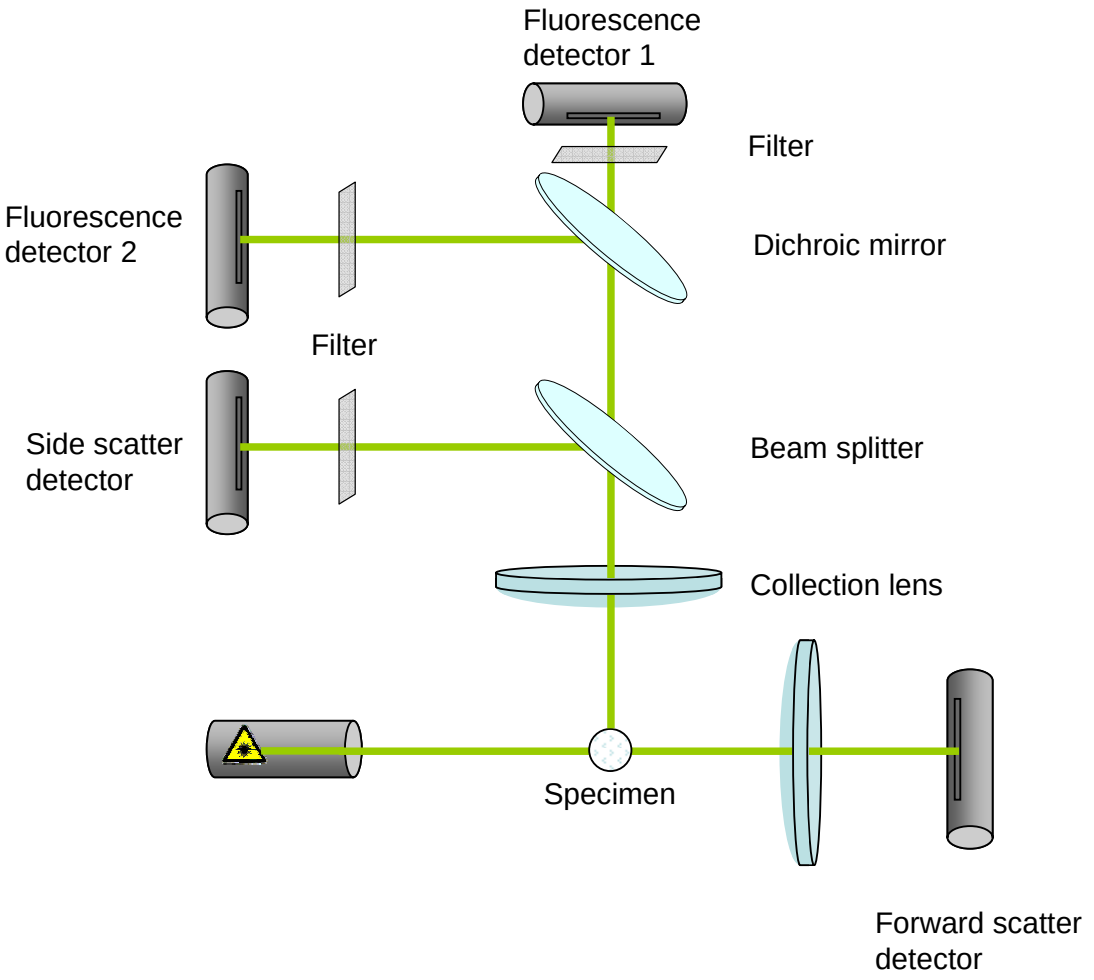


Figure 8: Basic optics of a flow cytometer.

6.1.2 Propidium iodide staining (Nicoletti)

Principle

Propidium iodide (PI) binds to DNA or RNA by intercalating between the bases with a stoichiometry of one per 4-5 base pairs (Waring, 1965). It can therefore be used to quantify the cell's DNA content. After binding to nucleic acids, its fluorescent characteristics are changed. Fluorescence is enhanced 20- to 30-fold, its excitation maximum is shifted approximately 40 nm to the red and the fluorescence emission maximum is shifted approximately 15 nm to the blue (Arndt-Jovin and Jovin, 1989). PI is membrane impermeable and generally excluded from viable cells. Thus, in order to determine the DNA content of

viable cells they have to be permeabilized using a specific PI-staining solution (HFS buffer).

Solutions

HFS Buffer			FACS buffer	
Propidium iodide		50.0 µg	Na-azide	0.20 g
Sodium citrate		0.1% (w/v)	KH2PO4	0.26 g
Triton X-100		0.1% (v/v)	KCl	0.28 g
PBS	ad	1000.0 µl	Na2EDTA	0.36 g
			LiCl	0.43 g
			Na2HPO4	2.35 g
			NaCl	8.12 g
			H2O	ad 1000 ml, pH 7.37

Experimental procedure

Cells were seeded with 50,000 cell/well in 6-well plates and kept in growth medium for 24 hours. Before stimulation, cells were serum-starved for four days to ensure synchronization in G_0-phase of the cell cycle.

For cell cycle analysis, cells were treated with 10% bovine serum or PDGF 20 ng/ml for different periods of time. CAPE was preincubated 30 min before stimulation with either serum or PDGF. After 48 hours, cells were trypsinized, washed once with PBS and resuspended in 500 µl PI-staining solution. After incubation in the dark at 4°C overnight, PI-stained nuclei were analyzed with a FACSCalibur (BD Biosciences).

6.1.3 BrdU/7-amino-actinomycin D (7-AAD) co-staining

Principle

After adding bromodeoxyuridine (BrdU) to the medium, it is incorporated in newly synthesized DNA instead of its analogue thymidine. Immunofluorescent staining of BrdU with specific FITC-labeled anti-BrdU fluorescent antibodies is used to determine the frequency and nature of individual cells that have synthesized DNA. Combining the immunofluorescent staining with applying a dye that binds to total DNA such as 7-AAD, permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) in terms of their cell cycle position (defined by 7-AAD staining intensities). Prolonged exposure of cells to BrdU allows for the identification and analysis of actively-cycling, as opposed to non-cycling, cell fractions. Pulse labeling of cells with BrdU at various time points, permits a detailed examination of cell-cycle kinetics. 7-amino-actinomycin D is used for determination of DNA content instead of PI in these experiments as the wavelengths of light emitted by 7-AAD and FITC does not overlap and can thus be analyzed in distinct channels.

Experimental procedure

Cells were seeded with 80,000 cells/well in 6-well plates and kept in growth medium for 24 hours. Before stimulation, cells were serum-starved for four days to ensure synchronization in G_0-phase of the cell cycle.

Cells were treated with 10% bovine serum for different periods of time. CAPE was preincubated 30 min before stimulation with either serum or PDGF. After 4 hours, 1 µM aphidicolin was added and cells were grown for another 15 hours in the presence of aphidicolin and 10% calf serum to achieve G_1/S-phase arrest. Subsequently, aphidicolin was removed by washing cells once with PBS before adding fresh growth medium containing 2 µM aphidicolin, 50 µM CAPE or vehicle for another 4 hours. For the last 30 minutes of incubation, cells were pulse labeled with 10µM BrdU, harvested and processed as described by the manufacturer (BrdU Flow Kit, BD Biosciences). Briefly, cells were fixed and permeabilized using buffers provided by the kit, followed by treatment with DNAse to expose BrdU epitopes. These were labeled using FITC-conjugated

anti-BrdU specific antibodies. Additionally, total DNA was stained with 7-AAD as described by the manufacturer. Analysis was performed with a FACSCalibur (BD Biosciences).

6.2 ^{3}H-thymidine incorporation

6.2.1 Principle

A second means to determine cells synthesizing DNA is the ^{3}H-thymidine incorporation. After adding radioactively labeled thymidine to the medium it is incorporated in newly synthesized DNA which becomes radioactive. Radioactive isotopes of hydrogen cannot be detected directly but by interaction of their radiation with the surrounding material. Due to its low energy content the β-radiation of ^{3}H-thymidine can not be measured by Geiger counters or similar devices but by using liquid scintillation counting (LSC). LSC is based on few well understood physical phenomena. The radioactive probe is dispensed homogenously in scintillation liquid. The kinetic energy is transformed into photons by exciting an appropriate fluorophore contained in the scintillation liquid. These photons are then converted into an electric pulse by a photovoltaic element. One of the major advantages of this method is the radioactive emitter being solved in the scintillation cocktail. The radiation can excite both the molecules of the solvent and the fluorophore. The molecules of the solvent in turn are able to transfer their energy to the fluorophore, too. Thereby the energy of the emitted radiation is transferred with almost 100 % to the fluorophore and no energy is lost in the transfer from emitter to scintillator. The total amount of excited fluorophores depends on the kinetic energy of the radioactive particle. The total quantity of photons generated is referred to as scintillation.

6.2.2 Experimental procedure

To measure DNA synthesis, VSMCs grown in 6-well plates were made quiescent for 48 hours in DMEM without calf serum. After pre-treatment with vehicle, CAPE or SB203580 at the indicated concentrations for 30 minutes, cells were treated with 20 ng/ml PDGF for 18 hours. Then 2 µCi/ml *methyl*-^{3}H-thymidine (Amersham, Biosciences, Freiburg, Germany)

were added, cells were pulse labeled for another 6 hours and the amount of incorporated ^{3}H-thymidine was assessed. In few words, cells were washed twice with ice-cold PBS and incubated with ice-cold 5% trichloracetic acid for 15 minutes at 4°C. After washing with 70% ethanol, the wells were dried. Then the cells were dissolved in 0.5 ml of 0.4 M NaOH for 15 minutes at room temperature. 0.4 ml of the NaOH solution were added to scintillation vials containing 0.2 ml of 1 N HCl. After adding 5 ml Liquiscint (Roth), the samples were analyzed in an LS 6500 Beckman Coulter (Beckman, Krefeld, Germany).

7 Microscopy

7.1 Confocal laser scanning microscopy (CLSM)

7.1.1 Principle

CLSM offers a higher optical resolution compared to conventional microscopy (figure 9). There are two major advantages. first, by a pinhole sitting conjugated to the optical plane (i.e. confocal) out-of-focus blurs are eliminated and the selectivity of the image is enhanced. Second, by using a laser it is possible to assemble a high resolution picture by scanning the sample sequentially, point by point with several information of every single pixel.

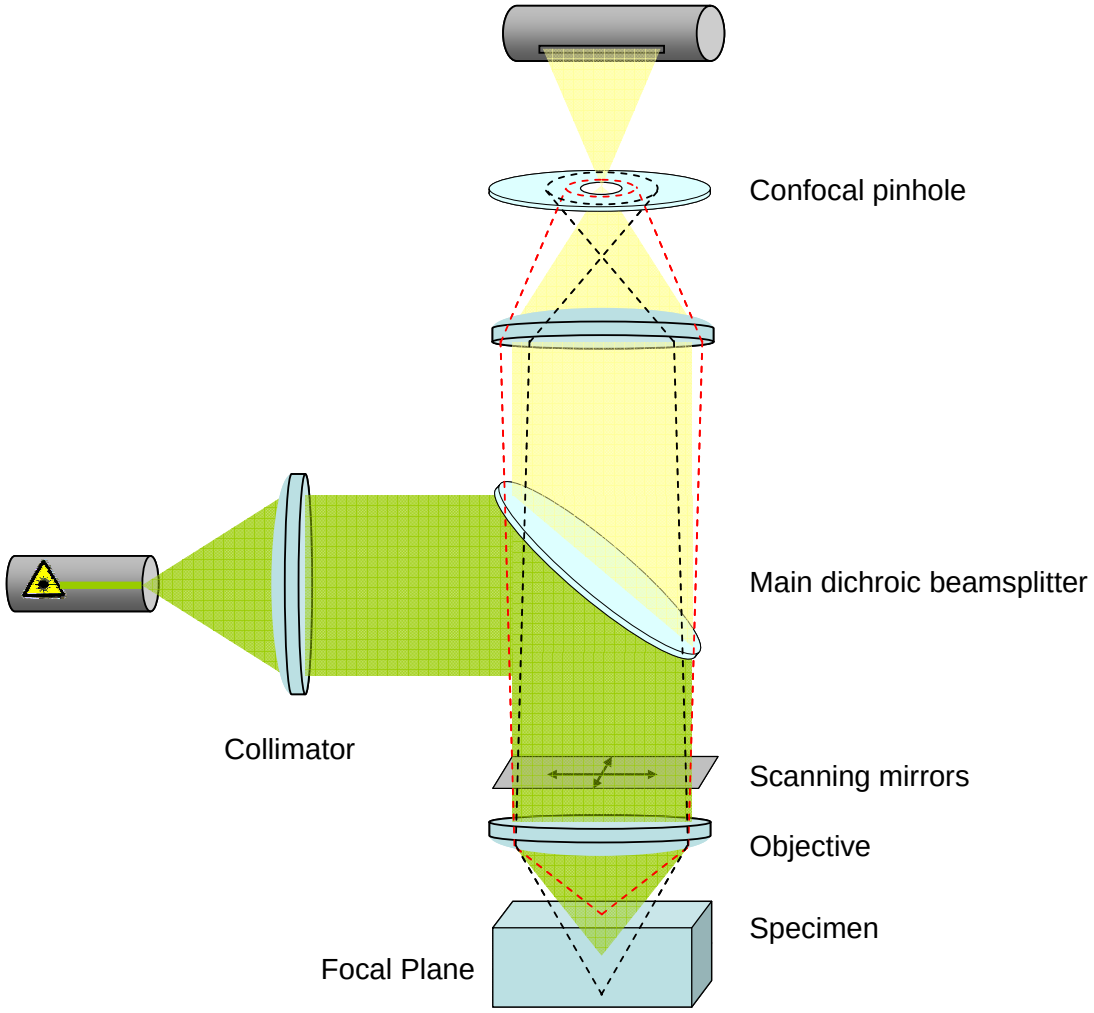


Figure 9: Principle of confocal laser scanning microscopy

7.1.2 Solutions

Fixative

Paraformaldehyde 4 g
PBS ad 100 ml

Dissolve paraformaldehyde by stirring and heating (max. 70°C) in a fume hood. After adding 1-2 drops of 1 N NaOH the solution was filtered and cooled down before use. The solution was freshly made before each experiment.

Blocking buffer

BSA		3 g
PBS	ad	100 ml

Quenching solution

NH_4C		0.078 g
PBS	ad	30.0 ml

7.1.3 Experimental procedure

Glass cover slips (∅ 12 mm) were prepared in 24-well plates. VSMC or mutant NIH 3T3 fibroblasts were grown to 50 % confluence and were treated as indicated in Results. After stimulation cells were washed twice with PBS and were fixed with fixative for 10 minutes at room temperature. Then cells were washed three times for 5 minutes and potential traces of paraformaldehyde were quenched with quenching solution for 10 minutes. Afterwards, the cells were rinsed once with PBS and then blocked for 1 hour with blocking buffer. Thereafter, the blocking buffer was removed and the appropriate primary antibody (table 4) was incubated for one hour. After the expiration of this time the cells were washed three times with PBS for 5 minutes and then the corresponding secondary antibody (table 5) was incubated for another hour. Finally, the cells were washed twice with PBS and once with PBS containing 5µg/ml Hoechst 33342, a fluorescent dye which intercalates between the DNA-strands, in order to stain the nucleus.

Table 4: primary antibodies

Target	Source	Dilution	Provider
Gab1	rabbit	1:100	Cell Signaling
PI3K-p85 N-SH2	mouse	1:100	Upstate

Table 5: secondary antibodies

Target	Source	Dilution	Provider
mouse IgG Alexa Fluor 633 (highly cross-absorbed)	Goat	1:400	Invitrogen
rabbit IgG Alexa Fluor 488	Goat	1:400	Invitrogen

8 Luciferase reporter gene assay

8.1 Principle

The activity of a promoter can be determined by a reporter gene assay. The promoter to be investigated, or a part of it, is inserted into a plasmid before a gene whose product can easily be both detected and quantified. The amount of protein synthesized by the transfected cell is dependent on the activity of the promoter. In our experiments we used firefly luciferase (from *Photinus pyralis*) under the control of the NF-κB promoter. Luciferase catalyzes the oxidation of luciferin to oxyluciferin using ATP and Mg^{2+} as co-substrates thereby emitting a photon (figure 10). This emission of light can be measured.

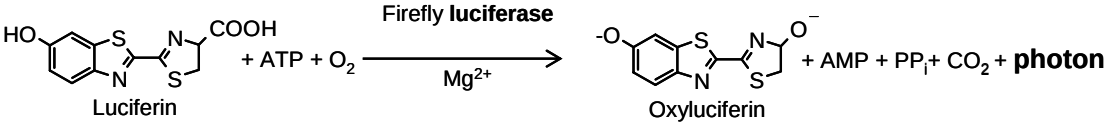


Figure 10: Luciferase catalyzed luminescence reaction

For normalization, cells are co-transfected with the gene encoding β-galactosidase. This *E.coli*-derived enzyme catalyzes the cleavage of the synthetic substrate chlorophenolred-β-D-galactopyranoside (CPRG) releasing chlorophenolred which is measured by spectrophotometry.

8.2 Transfection of HEK 293 cells

For the reporter gene assay human embryonic kidney 293 (HEK 293) cells were transiently transfected by using the calcium phosphate co-precipitation method originally described by Graham *et al.* (Graham and van der Eb, 1973) and modified by Jordan *et al.* (Jordan *et al.*, 1996) for the transfection of HEK 293 cells. By presenting the nucleic acid of the plasmids as a co-precipitate of calcium phosphate and DNA in the presence of salmon sperm, the uptake by cells grown in culture is significantly increased. After entering the cell by endocytosis, some of the co-precipitates escape from endosomes or lysosomes and enter the cytoplasm and are subsequently transferred to the nucleus. In order to check if the used method results in a transfection at all, the cells were firstly transfected with pEGFP-N1, a plasmid encoding for green fluorescent protein. This protein can easily be detected by fluorescent microscopy thus transfected cells can be determined in an easy way.

The ratio of cells transiently transfected with the gene varies from cell type to cell type and can reach up to 50%. The HEK cells were co-transfected with the luciferase reporter construct pNF-κB-Luc and the β-galactosidase gene pSV-β-Gal. The pSV-β-Gal plasmid contains the Simian virus 40 (SV40) promoter driving this gene. In order to exclude interactions between different plasmids, control cells were transfected with pNF-κB-Luc, pSV-β-Gal or salmon sperm alone. As positive control, the cells were co-transfected with pNF-κB-Luc, pSV-β-Gal, salmon sperm and pFC-MEKK. The latter encodes for MEKK which eventually results in NF-κB activation.

Table 6: List of Plasmids

name	promoter	reporter gene
pNF-κB-Luc	NF-κB (5x)	firefly-luciferase
pFC-MEKK	CMV	MEKK
pEGFP-N1	CMV	green fluorescent protein
pSV-β-Gal	SV40	lacZ

8.3 Solutions

Z-buffer

Na_2HPO_4	60 mM
NaH_2PO_4	40 mM
KCl	10 mM
$MgCl_2$	10 mM

Substrate solution

CPRG (50 mM)	100 µl
β-mercaptoethanol	10 µl
Z-buffer	10 ml

8.4 Experimental procedure

Cells were pre-incubated for 30 minutes with the indicated substances and subsequently stimulated with 1 ng/ml TNF-α for 6h. Then the cells were washed with PBS$^{\oplus}$ and lysed with the provided passive lysis buffer (Promega, Heidelberg, Germany). To ensure complete and equal coverage of the cells with the lysis buffer, the plates were placed on a rocking platform for 15 minutes at room temperature and frozen at -80°C until measurement. NF-κB activity was measured by the luciferase assay system (Promega, Heidelberg, Germany), according to the manufacturers description, with an AutoLumat Plus (Berthold, Pforzheim, Germany). Galactosidase activity was assayed by adding the substrate solution to 10 µl of each sample and measuring the absorbance at 550 nm at 37°C in a SpectraFluor Plus microplate reader (Tecan, Crailsheim, Germany).

9 Electrophoretic mobility shift assay (EMSA)

9.1 Principle

The interaction of proteins with DNA is crucial for many cellular processes including regulation of transcription. One established technique to study gene regulation and determining protein-DNA interactions is the electrophoretic mobility shift assay (EMSA). Nuclear extracts, containing the protein of interest, (here the transcription factor NF-κB) are incubated with linear DNA fragments consisting of the corresponding binding sequence. The principle of the EMSA is

based upon the fact that protein-DNA complexes migrate more slowly than free DNA when subjected to non-denaturing polyacrylamide gel electrophoresis.

9.2 Extraction of nuclear protein

Solutions:

Buffer A

HEPES (pH 7.9)	10.0 mM
KCl	10.0 mM
EDTA	0.1 mM
EGTA	0.1 mM
DTT	1.0 mM
PMSF	0.5 mM
Complete®	1.0 %

Buffer B

HEPES (pH 7.9)	20.0 mM
NaCl	400.0 mM
EDTA	1.0 mM
EGTA	0.5 mM
Glycerol	25.0 %
DTT	1.0 mM
PMSF	1.0 mM
Complete®	2.0 %

9.3 Experimental procedure

VSMCs were grown in 6-well plates. After pre-incubation with vehicle or CAPE for 30 minutes at the indicated concentrations, cells were treated with 10 ng/ml TNF-α for the denoted time. Thereafter, nuclear extracts were prepared as described by Schreiber *et al.* (Schreiber *et al.*, 1989) as follows: cells were washed with ice-cold PBS, scraped off in PBS with a rubber cell scraper, centrifuged, and resuspended in ice-cold hypotonic buffer A. Cells were incubated for 15 minutes. NP40 (10 %, 25 µl) was added to the cells followed by 10 seconds of vigorous vortexing and centrifugation of the homogenate at 14,000 g for 30 seconds. The supernatant, containing the cytosolic proteins, was removed. The nuclear pellet was resuspended by forceful shaking for 15 minutes at 4°C in hypertonic buffer B. The resulting nuclear extract was centrifuged at 14,000 g for 5 minutes and the supernatant containing nuclear proteins was frozen at -80°C. The protein concentrations were determined by the method of Bradford (Bradford, 1976).

9.4 Radioactive labelling of oligonucleotides

STE buffer (pH 7.5)

Tris-HCl	10 mM
NaCl	100 mM
EDTA	1 mM

9.4.1 Experimental procedure

Double-stranded oligonucleotide probes containing the consensus sequence for NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3') were 5'-end-labeled with adenosine 5'-[γ-^{32}P] triphosphate (3000 Ci/mmol) (Amersham, Freiburg, Germany) by using the T4 polynucleotide kinase (PNK) (USB, Cleveland, USA), which catalyzes the transfer of the terminal phosphate of ATP to the 5'-hydroxyl termini of DNA. The oligonucleotides were incubated with T4 PNK for 10 min at 37°C and the reaction was stopped by adding EDTA-solution (0.5 M). The radiolabeled DNA was separated from unlabeled remnants by using NucTrap probe purification columns (Stratagene, La Jolla, USA). Radiolabeled DNA was eluated from the column by STE buffer.

9.5 Binding reaction and electrophoretic separation

Solutions

Binding buffer (5x) (pH7.5)

Glycerol	20.0%
$MgCl_2$	5.0 mM
EDTA	2.5 mM
NaCl	250.0 mM
Tris-HCl	50.0 mM

Non-denaturing PAA-gel (4.5%)

TBE (10x)	5.30 %
Rotiphorese® Gel 30	15.80 %
Glycerol	2.60 %
TEMED	0.05 %
APS	0.08 %

Reaction buffer

DTT	2.6 mM
Binding buffer (5x)	90.0 %
Loading buffer	10 %

Loading buffer (pH7.5)

Tris-HCl	250.0 mM
Bromophenol blue	0.2 %
Glycerol	40.0 %

TBE buffer (10x) (pH 8.3)

Tris	0.89 M
Boric acid	0.89 M
EDTA	0.02 M

9.5.1 Experimental procedure

Equal amounts of nuclear protein were incubated for 5 minutes at room temperature (RT) in a total volume of 14 µl containing 2 µg poly(dIdC) and 3 µl reaction buffer. Subsequently, 1 µl of the radiolabeled oligonucleotide probe (approx. 300,000 cpm) was added. After incubation for 30 minutes (RT), the nucleoprotein-oligonucleotide complexes were separated by electrophoresis (Mini-Protean 3, Bio-Rad, Munich, Germany) on non-denaturing polyacrylamide gels (4.5 %). TBE was used as electrophoresis buffer. Bands were visualized by applying the gels to Cyclone Storage Phosphor Screens (Canberra-Packard, Dreieich, Germany) and analysis was perormed on a phosphor imager (Cyclone Storage Phosphor System, Canberra-Packard, Dreieich, Germany).

10 ^{1}H-Nuclear magnetic resonance (NMR) spectroscopy

10.1 Principle

The stability of a molecule is crucial for its biological function. CAPE is an unsaturated ester which can exist in two different isoforms. For all experiments it must be assured that the same isoform is used. NMR spectroscopy is one means to investigate potential changes in a molecule and to ensure a high purity. In this method the behavior of protons is observed. The chemical shift, i.e. the position of the signal in the spectrum, depends on how the proton is bound. The area under the corresponding signal is proportional to the number of protons interacting. The influence of adjacent protons can cause a splitting of the signal, referred to as spin coupling. By interpreting the chemical shift together with the area and a potential spin coupling effect, information of the properties and arrangement of the protons within the molecule can be obtained.

10.2 Experimental procedure

8 mg of CAPE were dissolved in 650 µl of water-free D^{6}-DMSO. The sample was transferred to an NMR-tube, measured and then frozen at -20 °C. Once a week, the probe was unfrozen and measured again. After 10 weeks, the solution was brought into sunlight for 1 hour and measured a last time as a positive control.

11 Cytotoxicity assays

In order to exclude that the observed effects are not due to cytotoxicity, experiments were performed to ensure that no toxicity was shown in the concentrations used.

11.1 Propidium iodide exclusion assay

11.1.1 Principle

Propidium iodide (PI) is a fluorophore which intercalates into DNA (see also chapter 6.1.2). When bound to nucleic acids, the absorption maximum for PI is 535 nm and the fluorescence emission maximum is 617 nm. PI can be excited with a xenon or mercury-arc lamp or with the 488 nm line of an argon-ion laser. Healthy cells with an intact membrane are not able to take up the dye while PI permeates membranes of dead cells. Hence, it is possible to distinguish viable from dead cells, which show an increased fluorescence signal.

11.1.2 Experimental procedure:

Cells grown in 6-well plates were serum-starved for 24 hours and treated with or without various concentrations of CAPE for 48 hours in the presence of 10 % calf serum. Cells were trypsinized, washed once with PBS and resuspended in 500 µl of PI solution. After incubation on ice in the dark, cells were analyzed by flow cytometry using the FL2 detector (FACSCalibur, BD Biosciences, Heidelberg, Germany).

PI solution

PI		2.5 µg
PBS	ad	1.0 ml

11.2 Trypan blue exclusion assay

11.2.1 Principle

The trypan blue exclusion assay is based on the same principle as the PI exclusion assay, except that the test is not based on DNA staining. Due to increased permeability of the membranes of dead cells, they can be stained with trypan blue and thus be differentiated from viable cells.

11.2.2 Experimental procedure

Cells grown in 6-well plates were serum-starved for 24 hours and treated with or without various concentrations of CAPE for 48 hours in the absence of calf

serum. Cells were trypsinized and analyzed in a Vi-Cell™ (Beckman Coulter, Krefeld, Germany)

12 Statistics

All experiments were performed at least three times. Results are expressed as mean ± standard error mean (S.E.M). Statistical analysis was performed by analysis of variance (ANOVA) followed by a Bonferroni multiple comparison test using Prism version 3.03 for Windows (GraphPad Software Inc., San Diego, USA) unless stated otherwise. P values <0.05 were considered significant.

D RESULTS

1 Influence of resveratrol on angiotensin II and EGF-treated VSMCs

In order to explain why the following experiments in this chapter have been performed, a brief review of our group's work will be given. Haider *et al.* have shown that RV is capable of reducing phosphorylation of Akt dose dependently. Further investigation of the pathway revealed that RV acts most likely *via* inhibition of PI3K to reduce Akt activation (Haider *et al.*, 2002). As described in chapter B 11.2 PI3K activation occurs by translocation to the plasma membrane by recruiting the SH2 domain of the p85 subunit to activated RTKs. Therefore, the signaling pathway of RTK transactivation and subsequent Akt activation was first investigated.

Experiments represented from D 1.1 to 1.4 were performed by Dr. Ursula Haider (Haider *et al.*, 2005). Experiment D 1.9 was performed by the group of B. Neel (Haider *et al.*, 2005). Since these results are important to understand the data generated within this thesis, they are implicated in the present work.

1.1 Resveratrol does not interfere with EGF-R transactivation.

To confirm previous observations showing that Ang II transactivates the EGF-R (Saito and Berk, 2001), we treated VSMCs with Ang II for various times and determined EGF-R tyrosine phosphorylation. Ang II caused a rapid increase in EGF-R phosphorylation that remained above baseline for 10 min (data not shown). We hypothesized that RV may interfere with this process. Agents previously described to act as inhibitors of transactivation (the antioxidant NAC, PP1, an inhibitor of c-Src kinase and the tyrphostin AG 1478, an inhibitor of EGF-R kinase) were used as positive controls. Figure 11 clearly shows that in contrast to NAC, PP1 and AG 1478, RV, at a concentration previously shown to inhibit Akt activation (Haider *et al.*, 2002), and figure 12A), does not influence Ang II-induced EGF-R transactivation.

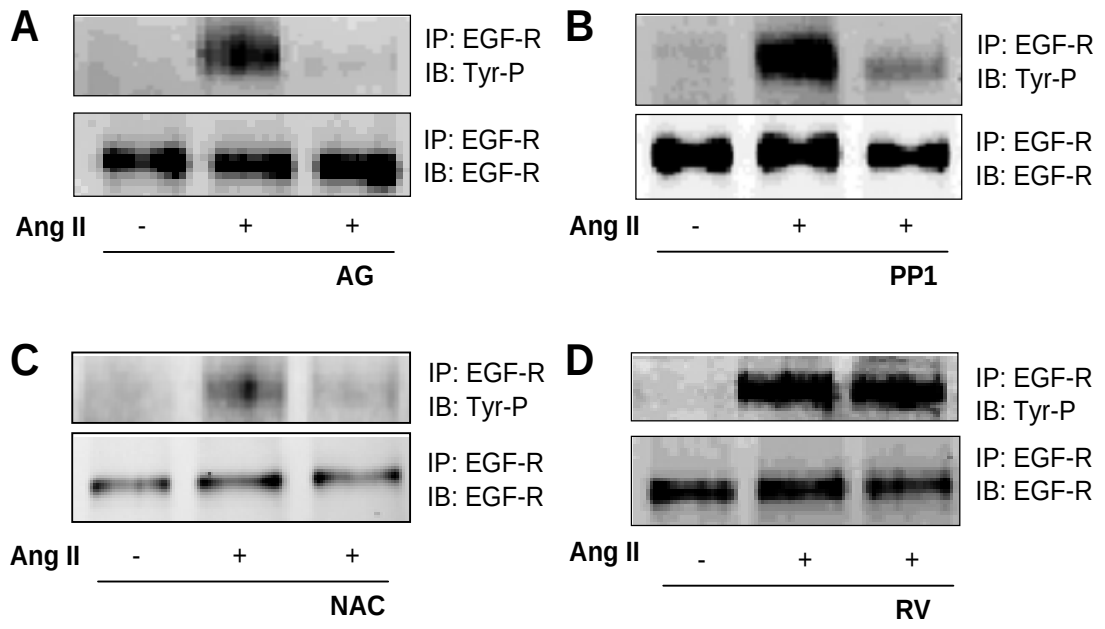


Figure 11. AG 1478, PP1 and NAC, but not RV, attenuate Ang II-induced EGF-R phosphorylation. VSMCs were preincubated with **A**, 250 nM AG 1478 (AG), **B**, 20 µM PP1, **C**, 10 mM NAC or **D**, 50 µM RV for 30 min before treatment with (+) or without (-) 100 nM Ang II for 2 min. Lysates were immunoprecipitated (IP) with anti-EGF-R antibody, followed by immunoblotting (IB) with anti-phospho-tyrosine (Tyr-P, top panel) or anti-EGF-R antibody (lower panel). Panels show one representative western blot out of three (Haider *et al.*, 2005).

1.2 Transactivation of EGF-R is important for Akt phosphorylation

Transactivation of the EGF-R has been shown to be essential for Akt activation by Ang II (Ushio-Fukai *et al.*, 2001b; Eguchi *et al.*, 1999b). To confirm that transactivation is indeed implicated in Akt phosphorylation in our cells, we treated VSMCs with AG 1478 prior to stimulation. The substance markedly decreased Akt phosphorylation (figure 12B), but failed to completely abolish the signal. These data indicate that EGF-R is indeed important for Ang II-induced Akt phosphorylation, although, to a smaller extent, parallel pathways should be considered.

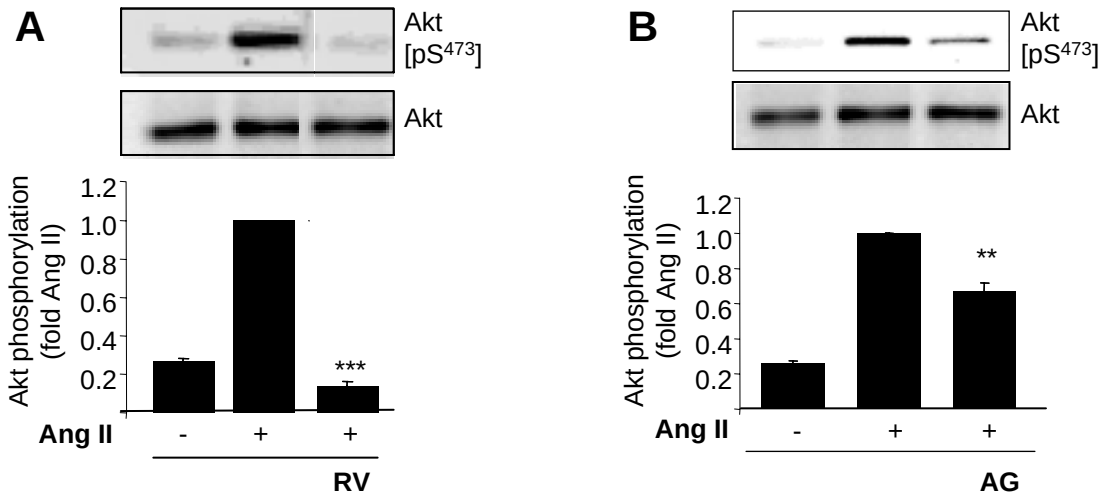


Figure 12. Effect of RV and AG 1478 on Ang II-induced Akt phosphorylation. VSMCs were preincubated with **A,** 50 µM RV, **B,** 250 nM AG 1478 (AG) or vehicle for 30 min before treatment with (+) or without (-) 100 nM Ang II for 10 min. Lysates were immunoblotted with anti-phospho-Akt (Ser473, top panels) or anti-Akt antibody (lower panels). Graphs at the bottom show mean ± SE from three independent experiments, expressed as inhibition of Ang II-induced Akt phosphorylation. **P<0.01 vs. Ang II; ***P<0.001 vs. Ang II (one-sample t test) (Haider *et al.*, 2005).

1.3 RV inhibits EGF-induced Akt phosphorylation

Since EGF-R transactivation is not impeded by RV, we assumed this substance to influence pathways downstream of the EGF-R. Consequently, we tested whether RV was also effective in blocking Akt phosphorylation when EGF instead of Ang II is used for stimulation. EGF rapidly stimulated Akt phosphorylation with a peak at 2 min (figure 13A). Figure 13B shows that RV as well as PP1, AG 1478 and wortmannin used as positive controls strongly inhibited EGF-induced Akt phosphorylation, suggesting that RV indeed interferes with pathways downstream of the EGF-R.

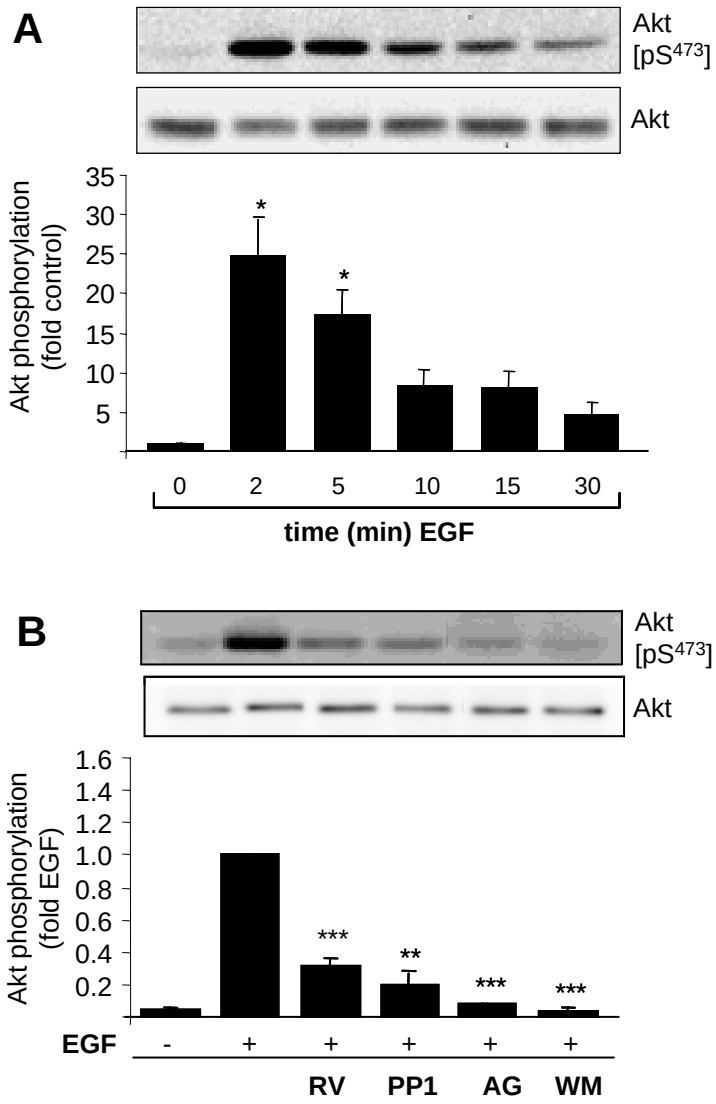


Figure 13. Effect of RV, AG 1478, PP1 and wortmannin on EGF-induced Akt phosphorylation. A, time course of EGF-induced Akt phosphorylation. VSMCs were treated with 100 ng/ml EGF for the indicated times. Lysates were immunoblotted with anti-phospho-Akt (Ser473, top panel) or anti-Akt antibody (lower panel). Graph at the bottom shows mean ± SE from three independent experiments, expressed as fold increase over control. *P<0.05 (ANOVA/Dunnett). **B**, VSMCs were preincubated with vehicle, 50 µM RV, 20 µM PP1, 250 nM AG 1478 (AG), or 50 nM wortmannin (WM) for 30 min before treatment with (+) or without (-) 100 ng/ml EGF for 4 min. Lysates were immunoblotted with anti-phospho-Akt (Ser473, top panel) or anti-Akt antibody (lower panel). Graph at the bottom shows averaged data, expressed as inhibition of EGF-induced phosphorylation. Values are mean ± S.E.M. from four independent experiments. **P<0.01 vs. EGF; ***P<0.001 vs. EGF (one-sample t test) (Haider *et al.*, 2005).

1.4 RV acts upstream of PI3K activation

PI3K is essential for Akt activation in both receptor tyrosine kinase- as well as G protein-coupled receptor-triggered pathways (Datta *et al.*, 1999). Ang II strongly activates PI3K activity in VSMCs, with concomitant phosphorylation of the kinase`s regulatory p85 subunit (Saward and Zahradka, 1997). Our group has previously shown that RV attenuates Ang II-stimulated tyrosine phosphorylation of the p85 subunit (Haider *et al.*, 2002). In contrast to wortmannin, however (figure 14A), RV failed to inhibit Akt phosphorylation upon calf serum stimulation (figure 14B). Taken together, these results argue against direct inhibition of PI3K activity by RV as shown for wortmannin (Wymann *et al.*, 1996).

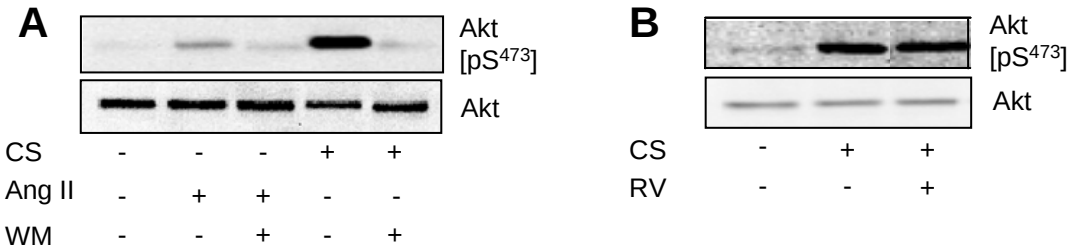


Figure 14. RV and wortmannin differently affect calf serum- and Ang II-induced Akt phosphorylation. **A**, impact of wortmannin (WM) on Ang II- and calf serum (CS)-induced Akt phosphorylation. Cells were treated with vehicle or 50 nM WM for 30 min and subsequently stimulated with (+) or without (-) CS (30 min) or Ang II (10 min). **B**, effect of RV on CS-induced Akt phosphorylation. Cells were treated with vehicle or 50 µM RV for 30 min before stimulation with (+) or without (-)10% CS for 30 min. Lysates were immunoblotted with anti-phospho-Akt (Ser473, top panels) or anti-Akt antibody (bottom panels) (Haider *et al.*, 2005).

1.5 RV decreases phosphorylation of Gab1 and binding of the PI3K p85 subunit to Gab1

The adapter molecule Gab1 has recently been shown to be involved in signal transduction from the EGF-R to PI3K. In a model proposed by Rodrigues *et al.*, Gab1 is first bound to and phosphorylated by the activated EGF-R before its interaction with PI3K leads to signal amplification by generation of PIP3. PIP3 recruits additional Gab1 and, subsequently, PI3K to the plasma membrane (Rodrigues *et al.*, 2000). In VSMCs, however, this link has never been

established. We therefore investigated whether EGF-stimulation a) enhances Gab1 tyrosine phosphorylation (figure 15A) and b) leads to the association of Gab1 with the PI3K regulatory p85 subunit (figure 15B). As both Gab1 tyrosine phosphorylation and p85-Gab1 association were strongly increased upon EGF-stimulation, we tested a possible influence of RV on these processes. Most interestingly, RV reduced both, overall Gab1 tyrosine phosphorylation (figure 15A) and association of Gab1 and p85 (figure 15B).

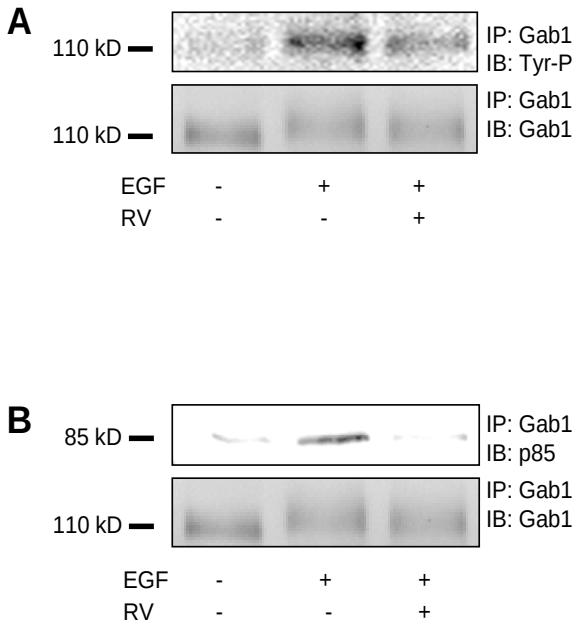


Figure 15. RV decreases phosphorylation of Gab1 and the binding of p85 to Gab1 in response to EGF. A, influence of RV on EGF-induced Gab1 phosphorylation. After pre-incubation with 50 µM RV (+) or vehicle (-) for 30 min, cells were stimulated with (+) or without (-) 100 ng/ml EGF (4 min). Cell lysates were immunoprecipitated with an anti-Gab1 antibody and subsequently immunoblotted with an anti-phospho-tyrosine antibody (upper panel) or anti-Gab1 antibody (lower panel). **B**, effect of RV on the binding of the regulatory subunit p85 of the PI3K to Gab1. VSMC were treated and cell lysates immunoprecipitated as described above. For immunoblot analysis an anti-p85 antibody (top panel) or anti-Gab1 antibody (lower panel) was used. One representative western blot out of three is shown.

1.6 RV decreases translocation of Gab1 and p85 to the plasma membrane

To confirm the result obtained by immunoprecipitation, we performed experiments by confocal microscopy (figure 16). According to the model mentioned above, EGF stimulation is associated with recruitment of Gab1 and p85 to the plasma membrane (Rodrigues *et al.*, 2000). Figure 16 shows that upon stimulation with EGF, both Gab1 and p85 translocate to the membrane. This process is potently inhibited when cells are preincubated with RV. Importantly, RV alone does not alter the distribution pattern of Gab1 and p85 compared to vehicle-treated control cells.

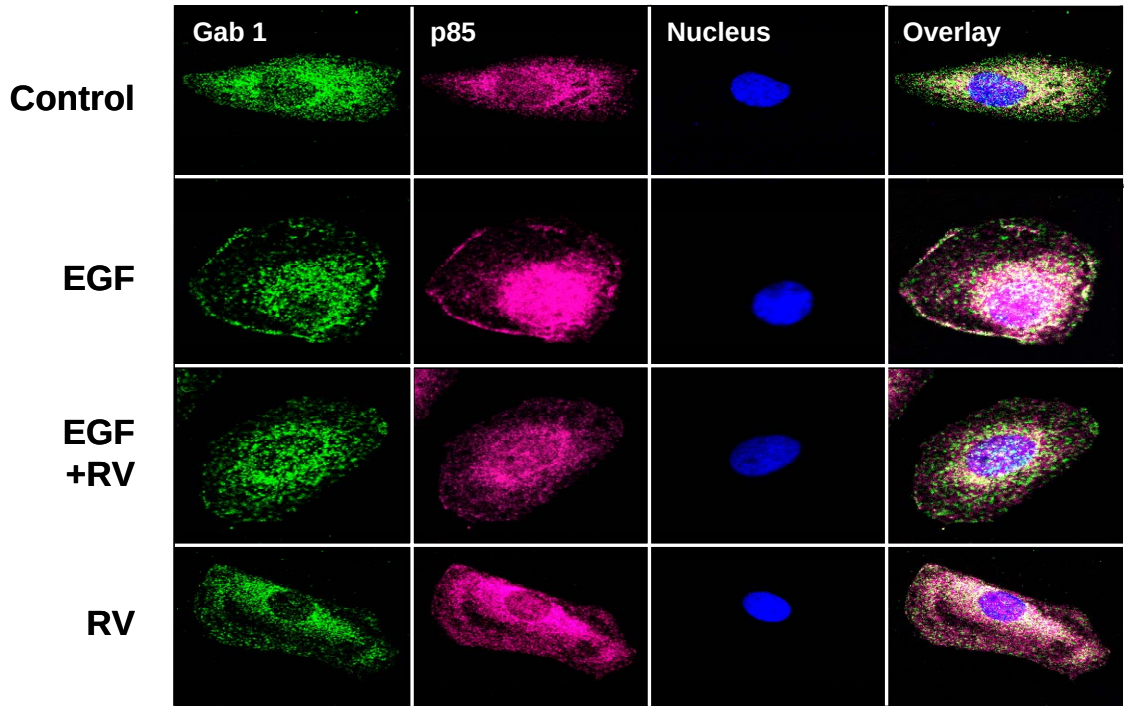


Figure 16. RV inhibits EGF-induced translocalization of Gab1 and p85 to the plasma membrane. VSMC were preincubated with vehicle or 50 µM RV for 30 min and subsequently stimulated with or without 100 ng/ml EGF for 4 min. After fixation with 4 % paraformaldehyde, cells were co-stained with anti-Gab1 antibody (green channel), anti-p85 antibody (pink channel) and Hoechst 33342 (blue channel) and analyzed by confocal microscopy.

1.7 RV decreases Ang II-induced Gab1 phosphorylation and PI3K p85 recruitment to Gab1

Next, we sought to clarify whether also in response to Ang II Gab1 tyrosine phosphorylation is increased and the PI3K p85 subunit is recruited to Gab1 in VSMC. Figure 17A/B clearly shows that both, Gab1 phosphorylation as well as PI3K p85-Gab1 association is stimulated in response to Ang II. RV inhibits both signaling events, although Gab1 phosphorylation to a lesser extend than p85-Gab1 association (figure 17A/B).

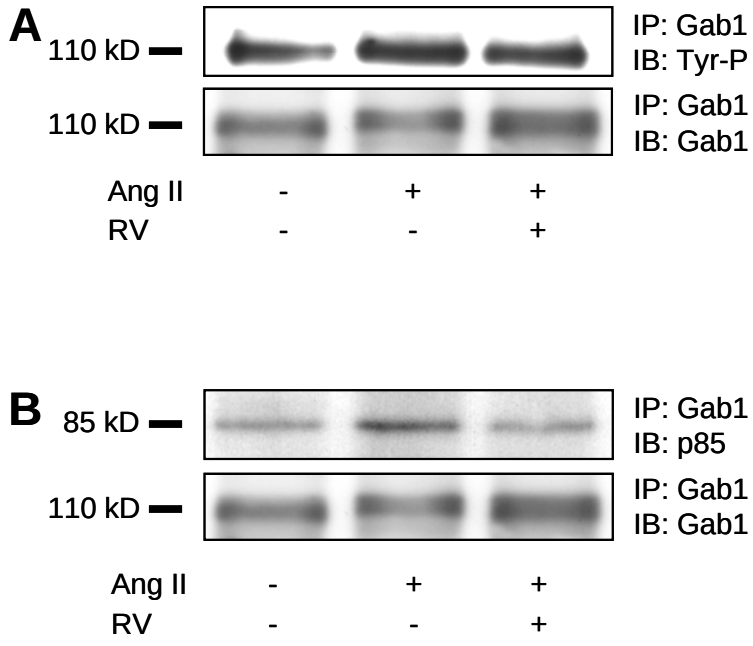


Figure 17. RV decreases phosphorylation of Gab1 and the binding of p85 to Gab1 in response to Ang II. **A**, influence of RV on Ang II-induced Gab1 phosphorylation. After pre-incubation with 50 µM RV (+) or vehicle (-) for 30 min, cells were stimulated with (+) or without (-) 100 nM Ang II (4 min). Cell lysates were immunoprecipitated with an anti-Gab1 antibody and subsequently immunoblotted with an anti-phospho-tyrosine antibody (upper panel) or anti-Gab1 antibody (lower panel). **B**, effect of RV on the binding of the regulatory subunit p85 of the PI3K to Gab1. VSMC were treated and cell lysates immunoprecipitated as described above. For Immunoblot analysis an anti-p85 antibody (top panel) or anti-Gab1 antibody (lower panel) was used. One representative western blot out of three is shown.

1.8 RV does not inhibit Akt activation in Shp-2$^{-/-}$-cells.

Recent evidence in fibroblasts revealed a major role for the phospho-tyrosine phosphatase Shp-2 in regulating Gab1/PI3K interaction. In these cells, inactivation of Shp-2 led to increased phosphorylation of the p85 binding sites on Gab1 and increased association of Gab1 and p85 upon EGF-stimulation with a subsequent increase in Akt activation (Zhang *et al.*, 2002). We therefore hypothesized that RV may act *via* activation of Shp-2. To verify this hypothesis, we used 3T3-immortalized fibroblasts from mice bearing a targeted mutation in Shp-2 exon 3 (Shp-2$^{\Delta 46\text{-}110}$, hereafter Shp-2$^{-/-}$) and Shp-2$^{-/-}$ fibroblasts where wild-type Shp-2 expression was restored by retroviral gene transfer at a level comparable to that found in wild-type 3T3 fibroblasts (thereafter, controls) (Zhang *et al.*, 2002). Western blot analysis revealed that RV did inhibit Akt activation upon EGF stimulation in restored control cells (figure 18B). However, in Shp-2$^{-/-}$ cells, RV failed to diminish EGF-induced Akt activation (figure 18A). Stimulation of Shp-2$^{-/-}$ fibroblasts and reconstituted control cells with Ang II and treatment with RV provided virtually the same result (data not shown). These data strongly indicate that RV action depends on the presence of functional Shp-2.

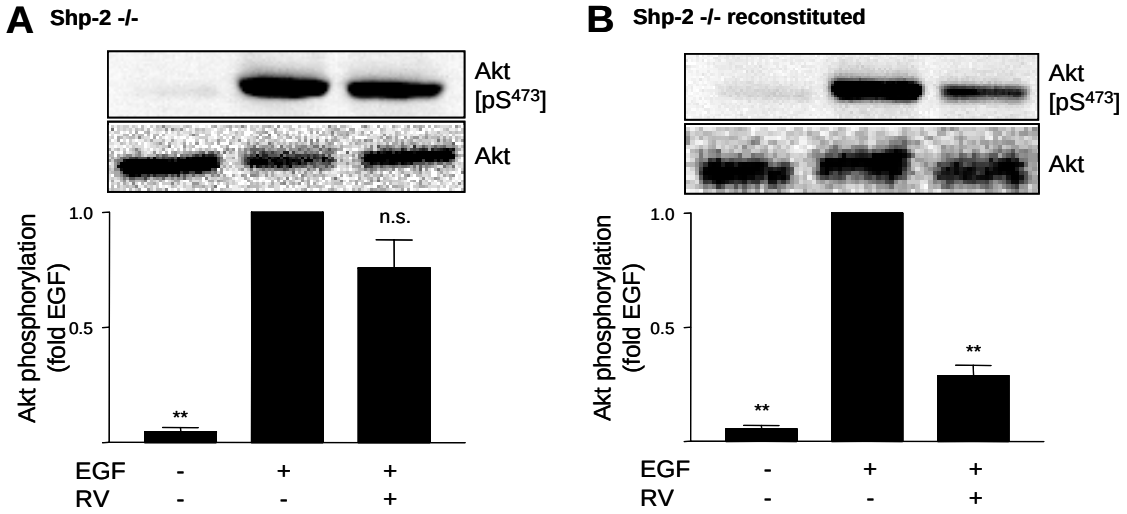


Figure 18. RV does not inhibit Akt phosphorylation in a hypomorphic Shp-2 mutant. A, 3T3 fibroblasts expressing hypomorphic mutant of Shp-2 (Shp-$2^{-/-}$) were treated with vehicle (-) or RV (+) for 30 min before stimulation with (+) or without (-) 100 ng/ml EGF for 2 min. Lysates were immunoblotted with anti-phospho-Akt (Ser473, top panel) or anti-Akt antibody (lower panel). **B**, 3T3 fibroblasts, reconstituted with wild-type Shp-2 were treated as described in A. **P<0.01 vs. EGF; n.s., not significant (Anova/Dunnett).

1.9 RV is capable of activating Shp-2

In order to gain deeper insight into the mechanism by which RV affects Shp-2, the Shp-2 activity was assayed in immune complexes from RV-treated cells (figure 19A) as well as the direct effects of RV on recombinant Shp-2 activity (figure 19B). RV-treated cells showed a dose-dependent (50 nM-5 µM) increase in Shp-2 activity in the presence of EGF, with a maximal stimulation of about 2.5-fold. RV had no statistical effect on Shp-2 activity, when EGF was absent (figure 19A). RV also induced a smaller (maximum <2-fold), but significant, direct dose-dependent increase in phosphatase activity of purified, recombinant Shp-2 (figure 19B). These data indicate that RV can activate Shp-2 phosphatase activity, in a dose-dependent manner, both in cell culture and *in vitro*.

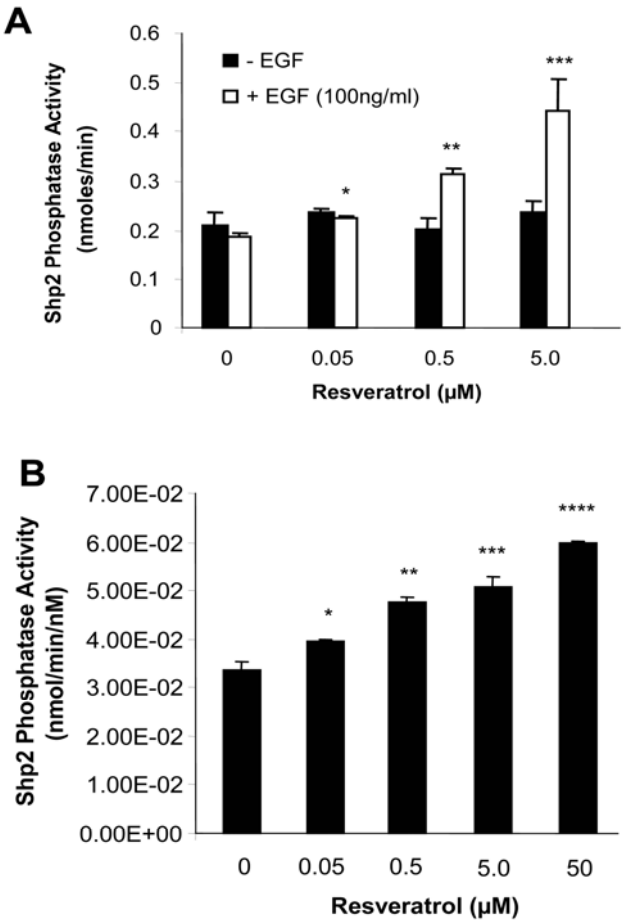
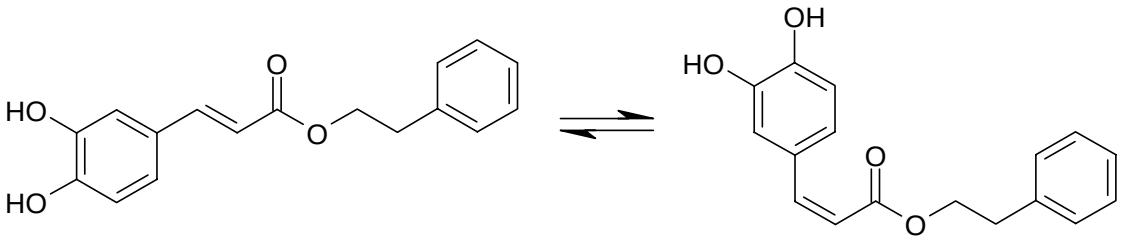


Figure 19 RV activates Shp-2. A, Shp-2 activity was measured in an immune complex phosphatase assay using Shp-2 rescue 3T3 fibroblasts. Briefly, Shp-2 reconstituted control cells were starved overnight, treated with various concentrations of RV for 30 min, and then stimulated with EGF (100 ng/ml) for 5 min. Cells were harvested and lysed in Nonidet P-40 buffer containing protease inhibitors, but no sodium orthovanadate. Following this lysates were immunoprecipitated with Shp-2 antibodies coupled to protein A-Sepharose beads. The amount of *para*-nitrophenyl phosphate (*p*NPP, Sigma-Aldrich) phosphate released was determined by measuring the absorbance at 410 nm. Results are means ± S.E. of the means from triplicate, and data were analyzed by a one-tailed paired t test. *, P = 0.04 **, P = 0.002; ***, P = 0.03. RV had no statistical significance on Shp-2 activity in the absence of EGF. **B**, Shp-2 phosphatase activity was measured in an *in vitro* assay using purified recombinant Shp-2 protein (10 nM). The rate of hydrolysis of pNPP by Shp-2 was measured in buffer containing various concentrations of RV. Results are means ± S.E: of the means from triplicate, and data were analyzed by a one-tailed paired t test. *, P = 0.045; **, P = 0.02; ***, P = 0.0003; ****, P = 0.003.

2 Influence of CAPE on calf serum- and PDGF-treated VSMCs

2.1 Stability of CAPE

CAPE exists in two isomers (figure 20) from which we used *trans*-CAPE. Its conformation can be changed upon light exposure.



***trans*-CAPE** ***cis*-CAPE**

Figure 20. CAPE isomers.

As we used a stock solution of *trans*-CAPE it was of interest to know whether CAPE is stable during usage. In order to confirm its photo stability, CAPE was analyzed by ^{1}H-NMR spectroscopy as described in materials and methods. Figure A shows the NMR spectrum of freshly solved CAPE, figure B shows CAPE after 10 weeks. As can be clearly seen there is no difference between the two spectra A and B whereas the spectrum in figure C shows noticeable extra peaks resulting from the mixture of the two isoforms. This conformational change was achieved by one hour of intensive sunlight exposure to the solution. These data confirm that dissolved *trans*-CAPE is stable during normal usage but unstable when exposed to light.

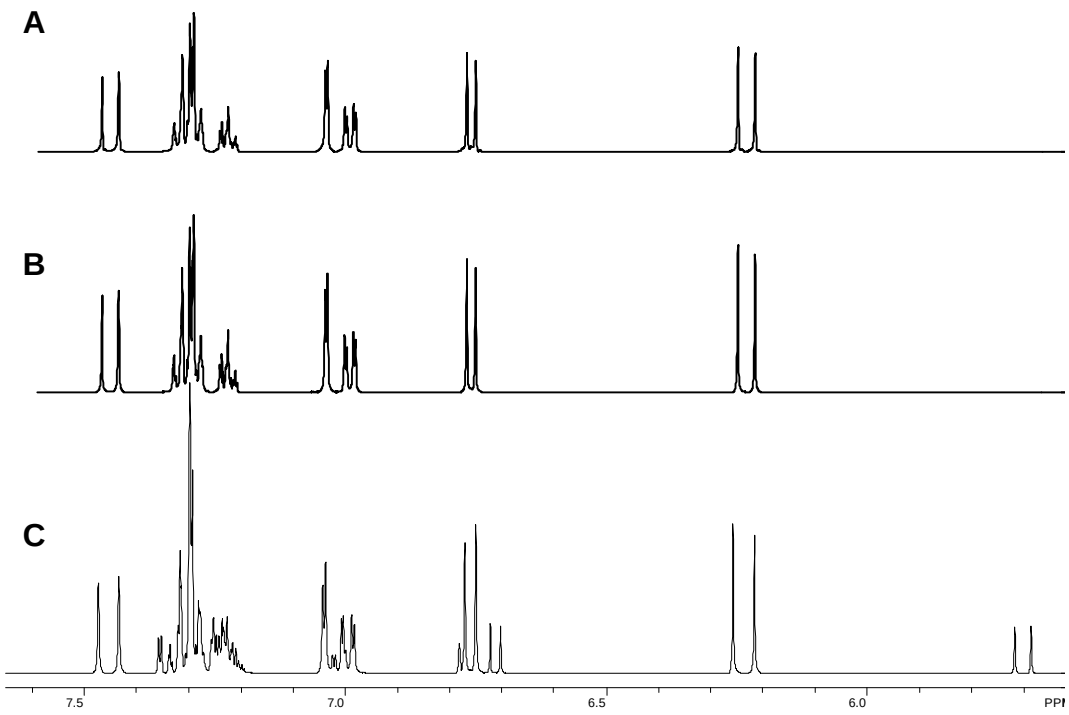


Figure 21. *trans*-CAPE is stable in normal use but alters conformation upon light exposure. A, After dissolving of 8 mg CAPE in water-free D^6-DMSO the sample was measured and then frozen under the same conditions as the working stock (-20 °C). **B,** after ten weeks and ten freeze-thaw cycles the probe was measured again. **C,** after exposure to sunlight for one hour the sample was measured a last time.

2.2 CAPE dose-dependently inhibits serum-induced VSMC proliferation

Treatment of VSMCs with different concentrations of CAPE (1-50 µM) caused a dose-dependent inhibition of serum-induced (10%) cell proliferation (figure 22). Cell number of the serum group increased to about 17 fold compared to vehicle-treated control cells after 72 h. CAPE inhibited serum-induced cell growth completely at a concentration of 25 µM.

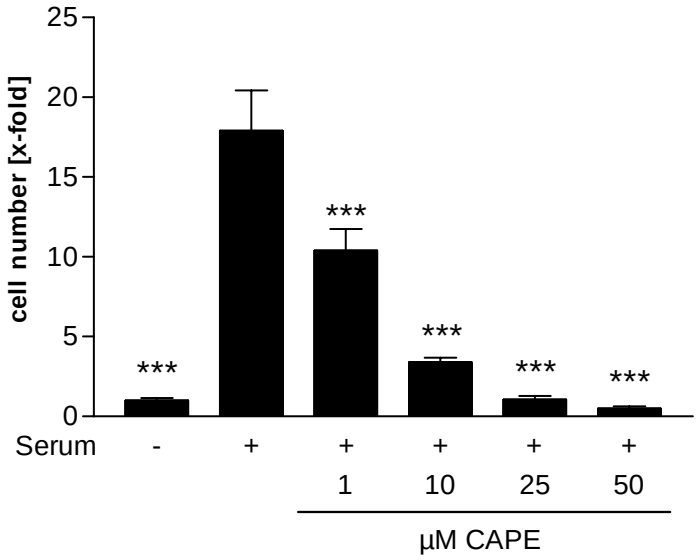


Figure 22. VSMC proliferation upon serum-stimulation is inhibited dose-dependently by CAPE. After serum withdrawal for 48 h cells were treated with CAPE at the indicated concentrations or vehicle for 30 minutes prior to stimulation with 10% serum (+) for 72 h. After trypsination, the cell number was assessed by cell counting; unstimulated control values (-) were set as 1. The histogram shows the means ±S.E.M of cell numbers compared to serum of at least three independent experiments. ***, $p < 0.001$ versus PDGF (ANOVA/Bonferroni).

2.3 CAPE arrests the cell cycle of serum stimulated VSMC in S-phase

Treatment of serum-stimulated (10%) VSMCs with 50 µM CAPE leads to a cell cycle arrest (figure 23).

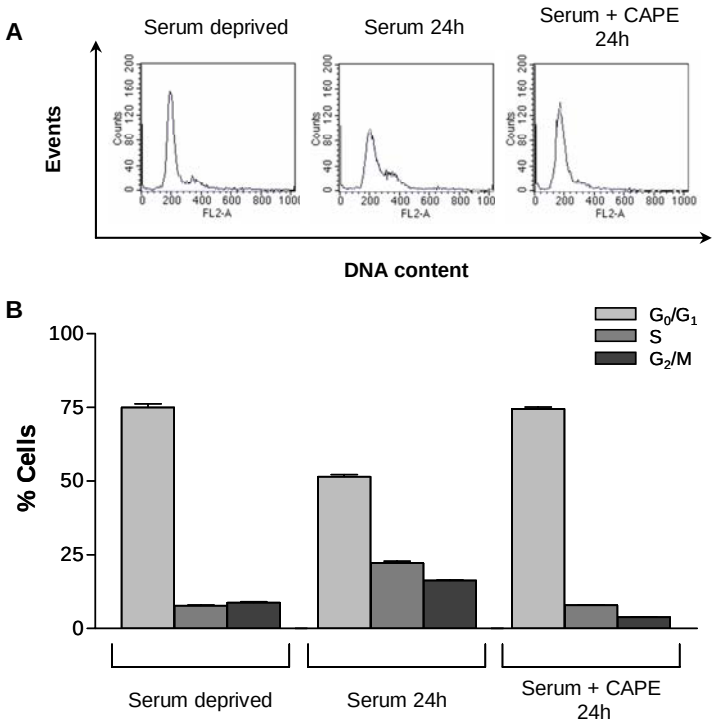


Figure 23. After pre-incubation with vehicle or 5 µM CAPE for 30 minutes, VSMCs were stimulated with 10% serum for 24 h. Cell cycle phase distribution was determined by flow cytometry after propidium iodide staining of DNA as described in materials and methods. **A**, Representative histograms of flow cytometry are shown. **B**, Quantification of cells in G0/G1, S and G2/M-phase. Results are means ±S.E.M. from three independent experiments.

In order to confirm this assumption a BrdU incorporation assay as described in materials and methods was performed. To synchronize the cells in early S-phase, aphidicolin was added. Aphidicolin binds directly to DNA polymerase and blocks DNA synthesis, while allowing for RNA and protein synthesis and progression through G1 (Pedrali-Noy *et al.*, 1980). In accordance with the PI-staining assay (figure 23 A), CAPE is capable of inhibiting cell cycle progression. The shape of the CAPE dot blot resembles that of aphidicolin, hence it is very likely that CAPE arrest the VSMCs in S-phase (figure 24).

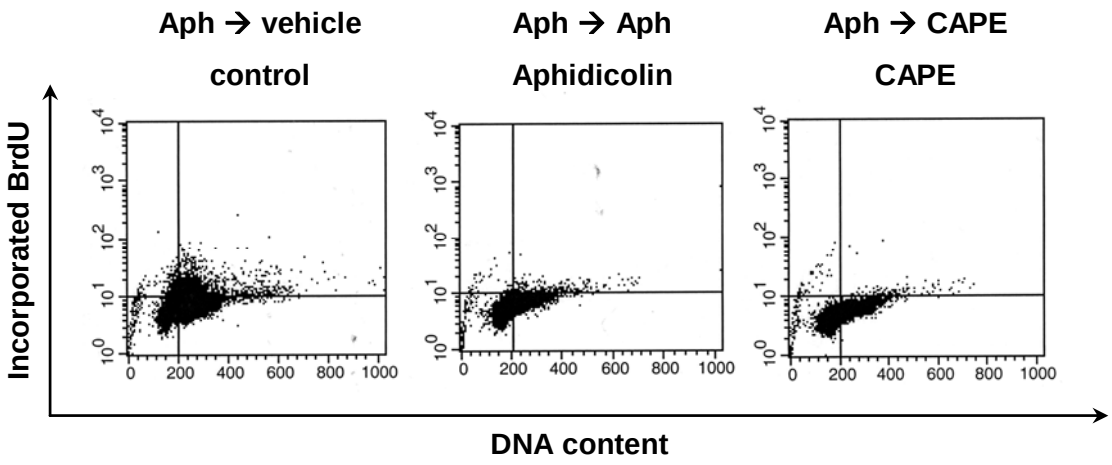


Figure 24. CAPE causes a cell cycle arrest at G_1/S. After synchronization of the cells with 1 µM aphidicolin for 15 h with, cells were washed once with PBS. Subsequently, fresh growth medium containing 2 µM aphidicolin, 50 µM CAPE or vehicle was added for another 4 hours. For the last 30 minutes of incubation, cells were pulse labeled with 10µM BrdU, harvested and processed as described in material and methods. Representative dot blots of flow cytometry are shown. The total DNA content, visualized *via* 7-AAD, is shown on the x-axis, while newly synthesized DNA, visualized *via* BrdU incorporation, is depicted on the y-axis.

2.4 CAPE shows no toxicity on serum-cultured but on serum deprived VSMCs

In order to ensure that the anti-proliferative effects of CAPE are not due to cytotoxicity two experiments were performed. First, a PI exclusion assay was performed with both serum-treated and serum deprived VSMCs. As shown in figure 25 serum-treated cells show no cytotoxic effect of CAPE whereas the membranes of serum-deprived cells become PI permeable, an evidence for cytotoxicity.

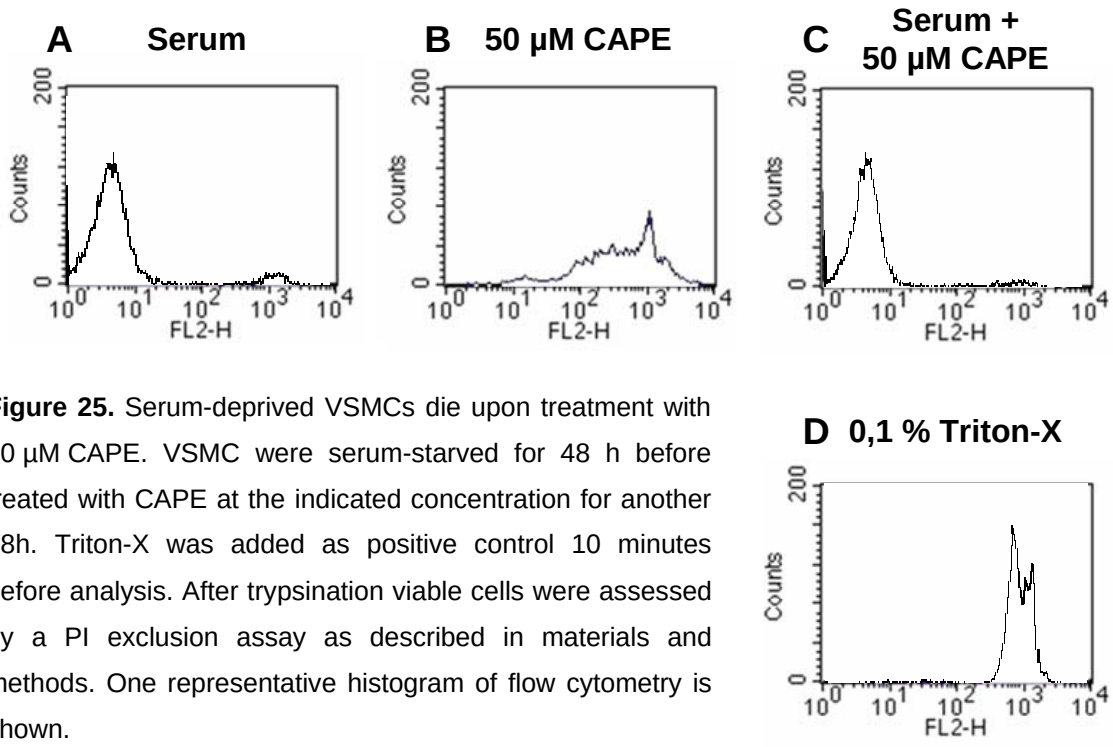


Figure 25. Serum-deprived VSMCs die upon treatment with 50 µM CAPE. VSMC were serum-starved for 48 h before treated with CAPE at the indicated concentration for another 48h. Triton-X was added as positive control 10 minutes before analysis. After trypsination viable cells were assessed by a PI exclusion assay as described in materials and methods. One representative histogram of flow cytometry is shown.

The second experiment, a trypan blue exclusion assay, was performed to determine the cytotoxic concentration of CAPE on serum-deprived cells. As can be seen in figure 26, only in concentrations higher than 25 µM cytotoxicity of CAPE could be observed.

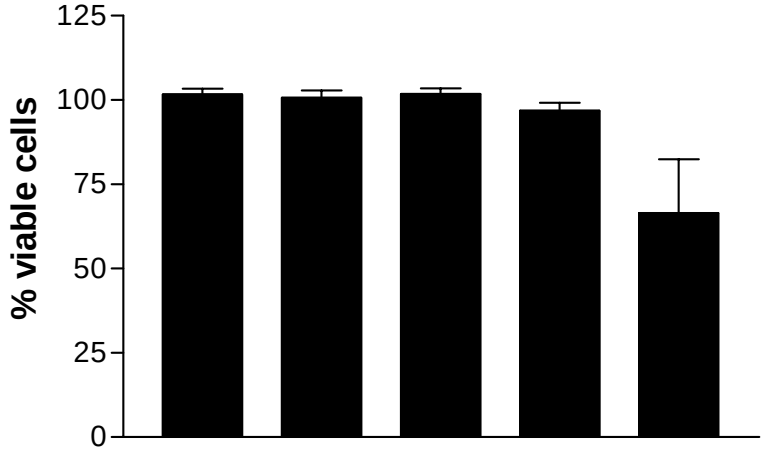


Figure 26. Cell viability. VSMC were serum-starved for 48 h before treatement with CAPE at the indicated concentration for another 48 h. After trypsination, the viable cell number was assessed by cell counting in a trypan blue assay; Control values were set as 100%. The histogram shows the means ±S.E.M of cell numbers compared to vehicle-treated cells of at least three independent experiments.

2.5 CAPE dose-dependently inhibits PDGF- induced VSMC proliferation

Serum is a multi-component stimulus, therefore, it is very difficult to elucidate the pathways involved in the before mentioned growth inhibitory effect of CAPE. As PDGF-BB is a potent stimulus for VSMC proliferation it was chosen to investigate the signaling pathway.

Treatment of VSMCs with different concentrations of CAPE (1-10 µM) caused a dose-dependent inhibition of PDGF-BB (20 ng/ml)-induced cell proliferation (figure 27 A). Cell number of the PDGF-group increased to about 1.4 fold compared to vehicle-treated control cells after 48 h. CAPE blocked PDGF-induced cell growth completely at a concentration of 5 µM. To confirm these finding we measured DNA synthesis by ^{3}H-thymidine incorporation. In accordance with cell counting results, CAPE (5 µM) was capable to reduce the

amount of PDGF-induced thymidine incorporation to almost control levels (figure 27 B).

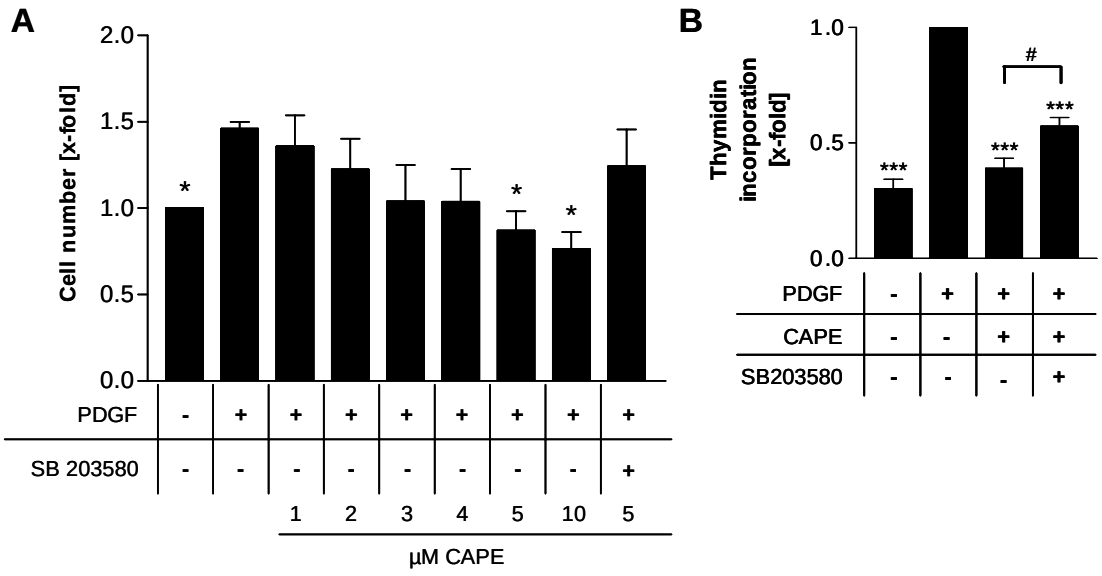


Figure 27. CAPE dose-dependently impedes PDGF-induced proliferation of VSMCs. A, Cells were treated with vehicle (-), 5 µM SB203580 (+) or CAPE at the indicated concentrations for 30 minutes before stimulation with (+) or without (-) 20 ng/ml PDGF-BB for 48 h. After trypsination, the cell number was assessed by cell counting; Control values were set as 1. The histogram shows the means ± S.E.M of cell numbers compared to PDGF of at least three independent experiments. **B**, VSMCs were pre-treated with vehicle (-), 5 µM CAPE or 5 µM SB203580 for 30 minutes, before stimulation with (+) or without (-) 20 ng/ml PDGF-BB for 24 h. Thymidine incorporation was assessed as described in materials and methods. The histogram shows means of three independent experiments whereas PDGF values were set as 1. *, $p < 0.05$; ***, $p < 0.001$ versus PDGF (ANOVA/Bonferroni). #, $p < 0.05$ versus PDGF + CAPE (ANOVA/Bonferroni)

2.6 Treatment of VSMCs with CAPE results in amplified and sustained p38 MAPK activation that contributes to the antiproliferative effect

Activation of VSMC with PDGF-BB led to induction of several growth-related signaling pathways, such as the MAPK (p38, ERK1/2), and the PI_3K/Akt pathway (figure 28 A) which is in accordance with published data (Zhan *et al.*, 2003). None of these kinases, however, was inhibited by CAPE at concentrations effectively blocking PDGF-induced cell proliferation. In contrast, in the presence of PDGF-BB CAPE (5 µM) caused a strengthened and prolonged activation of p38 MAPK compared to PDGF-BB alone (figure 28 B/C). Therefore, we employed the p38 inhibitor SB203580 (5 µM) to see whether this sustained activation of p38 can be linked to the antiproliferative activity of CAPE. As can be seen in figure 27 A/B, SB203580 indeed partly neutralized the inhibitory effect of CAPE indicating that p38 at least contributes to the antiproliferative effect of CAPE.

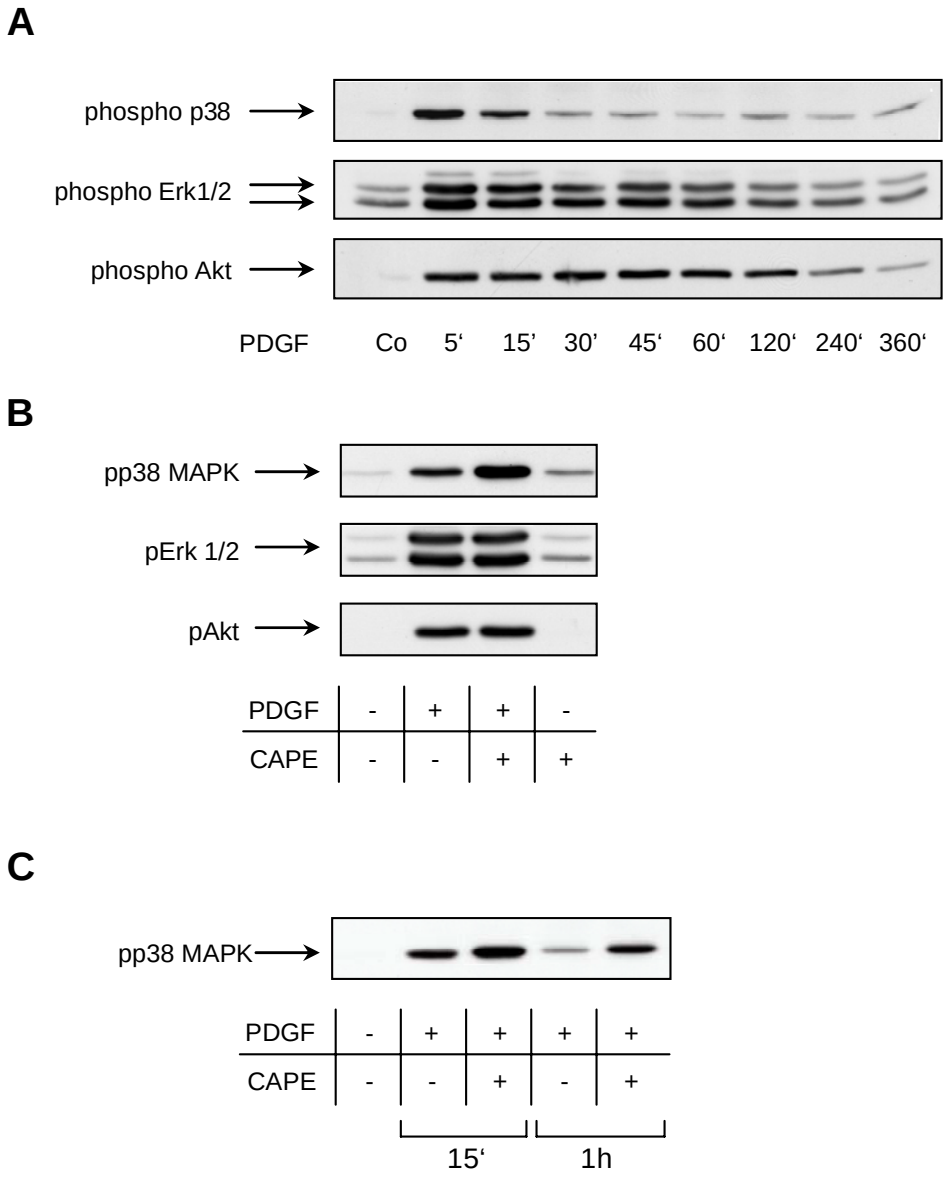


Figure 28. PDGF induces several growth-related kinases but CAPE influences only p38 MAPK. A, Time course of phosphorylation of growth-related kinases p38, Erk1/2, Akt in response to PDGF treatment. Cells were treated with PDGF-BB (20 ng/ml) for the indicated time and lysed for Western blot analysis as described in materials and methods. **B**, Influence of CAPE on PDGF-induced phosphorylation of p38, Erk1/2, and Akt. After pre-treatment with 5 µM CAPE (+) or vehicle (-) for 30 minutes, cells were stimulated with 20 g/ml PDGF-BB for 10 minutes. Cell lysates were immunoblotted with an anti phospho-p38, anti phospho-Erk1/2, and anti phospho-Akt antibody. **C**, Time course of p38-phosphorylation upon PDGF-BB stimulation with or without CAPE pre-incubation. Cell were treated with 5 µM CAPE (+) or vehicle (-) for 30 minutes before stimulation with 20 ng/ml PDGF-BB for the indicated time. Cell lysates were immunoblotted with an anti phospho-p38 antibody. One representative Western Blot out of three is shown, respectively.

2.7 CAPE influences cell cycle progression of PDGF-stimulated VSMCs

In order to specify the antiproliferative effect of CAPE and to additionally exclude cytotoxic effects, the cell number in each cell cycle phase was determined *via* flow cytometry (FACS). After exposure of VSMCs to 5 µM CAPE in the presence or absence of PDGF-BB for 28 h, FACS analysis showed that the proportion of cells in G0/G1-phase increased and the proportion of S- and M-phase decreased compared to PDGF-BB-treated cells (figure 29 A/B) indicating that CAPE-treated cells tend to accumulate in G0/G1-phase. No sub-G1-peak was observed excluding apoptosis induction in response to CAPE.

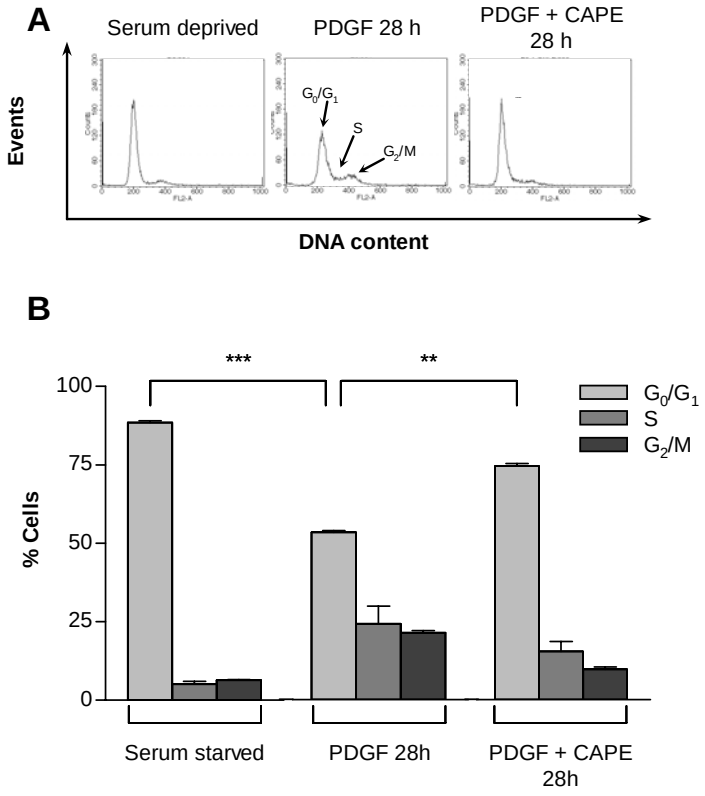


Figure 29. CAPE increases number of cells in G1 phase in PDGF-activated VSMC. After pre-incubation with vehicle or 5 µM CAPE for 30 minutes, VSMCs were stimulated with PDGF-BB (20 ng/ml) for 28 h. Cell cycle phase distribution was determined by flow cytometry after propidium iodide staining of DNA as described in materials and methods. **A**, Representative histograms of flow cytometry are shown. **B**, Quantification of cells in G0/G1, S and G2/M-phase. Results are means ±S.E.M. from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$ versus G0/G1 phase (ANOVA/Bonferroni)

2.8 The antiproliferative effect of CAPE is not mediated by NF-κB

CAPE is a well known inhibitor of the transcription factor NF-κB (Natarajan *et al.*, 1996). In addition, NF-κB was shown to regulate cell cycle progression and proliferation of a number of cell types, including VSMC, although seemingly dependent on the stimulus used (Mehrhof *et al.*, 2005; Selzman *et al.*, 1999). We, therefore, tested whether the antiproliferative effect of CAPE might be mediated by inhibition of this transcription factor. As demonstrated in figure 30 A, CAPE (5 µM) was not able to reduce the NF-κB binding activity determined by an electrophoretic mobility shift assay (EMSA). Also NF-κB transactivation activity was not impaired by 5 µM CAPE as shown by a luciferase reporter gene assay (figure 30 B). Altogether, we found that CAPE does not inhibit NF-κB at 5 µM but at higher concentrations (50 and 75 µM).

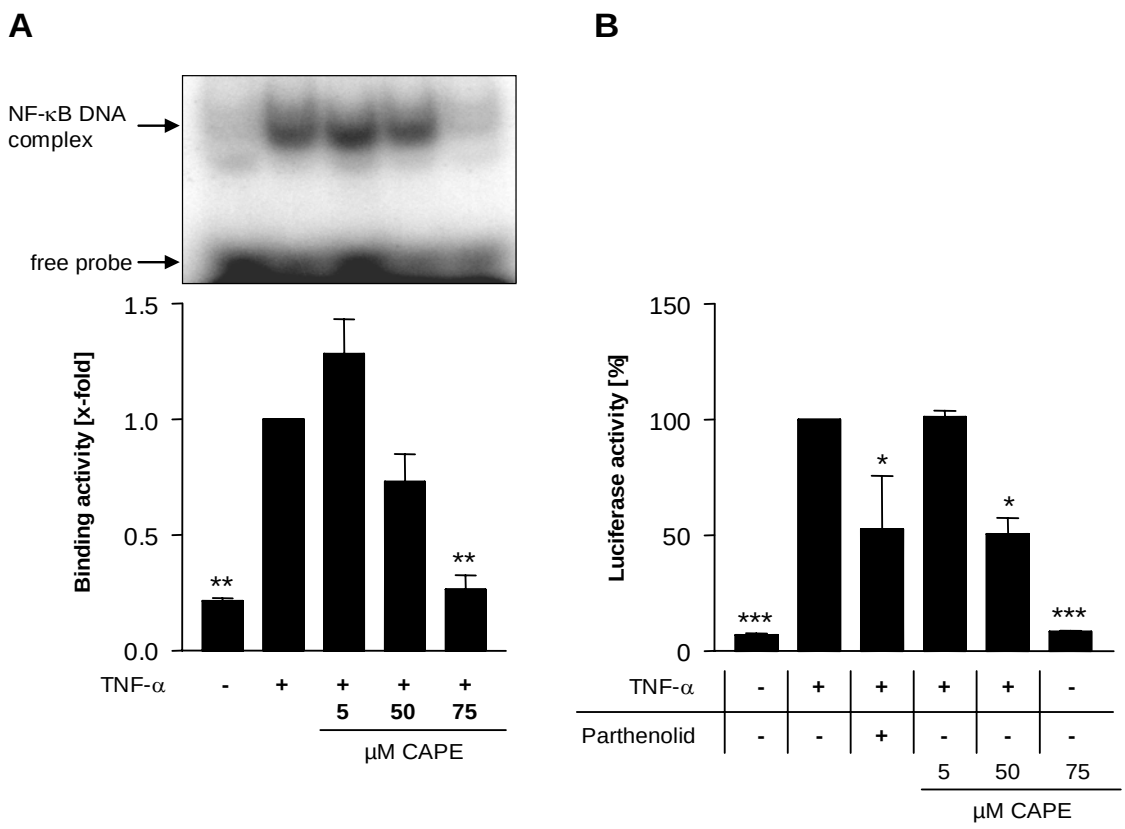


Figure 30: CAPE does not affect NF-κB activity at a concentration of 5 μM. A, VSMCs were pre-treated with vehicle or CAPE at the indicated concentrations for 30 minutes before stimulation with 10 ng/ml TNF-α for 15 minutes. NF-κB binding activity was measured by electrophoretic mobility shift assay (EMSA) as described in materials and methods. Data show one representative out of three independent experiments from different cell preparations with similar results. The histogram shows phosphor imaging analysis of EMSA experiments and represents means ±S.E.M.; values of TNF-α treated cells were set as 1. **B**, Transiently transfected HEK 293 cells containing the luciferase reporter construct pNF-κB-Luc and the β-galactosidase gene pSV-β-Gal were pre-treated with vehicle or CAPE at the indicated concentrations for 30 minutes before stimulation with 10 ng/ml TNF-α. The histogram shows luminometer analysis of three independent experiments and represents means ±S.E.M. Values of TNF-α treated cells were set as 1.*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (ANOVA/Bonferroni).

2.9 HO-1 induction by CAPE seems not to be involved in the antiproliferative effect

CAPE is described as a heme oxygenase-1 (HO-1) inducer in astrocytes (Scapagnini *et al.*, 2002). In addition, HO-1 is known to lead to cell cycle arrest in VSMCs (Choi *et al.*, 2004; Duckers *et al.*, 2001). Moreover, carbon monoxide (CO), a product of HO-1 activity was recently shown to mediate its antiproliferative effect *via* p38 activation (Kim *et al.*, 2005). We, therefore, investigated whether CAPE induces HO-1 in VSMCs and whether this putative induction may contribute to the antiproliferative effect of CAPE in VSMC. As shown in Figure 31 A both, cobalt protoporphyrin (CoPP, 10 µM) used as positive control and CAPE (5 µM) caused a potent increase in HO-1 expression in VSMCs which is not influenced by the p38 inhibitor SB203580. To examine whether the antiproliferative effect of CAPE may by due to induction of HO-1, we repeated cell counting experiments employing SnPP 10 µM as HO-1 inhibitor. Figure 31 B indicates that HO-1 does not contribute to the CAPE induced cell cycle arrest.

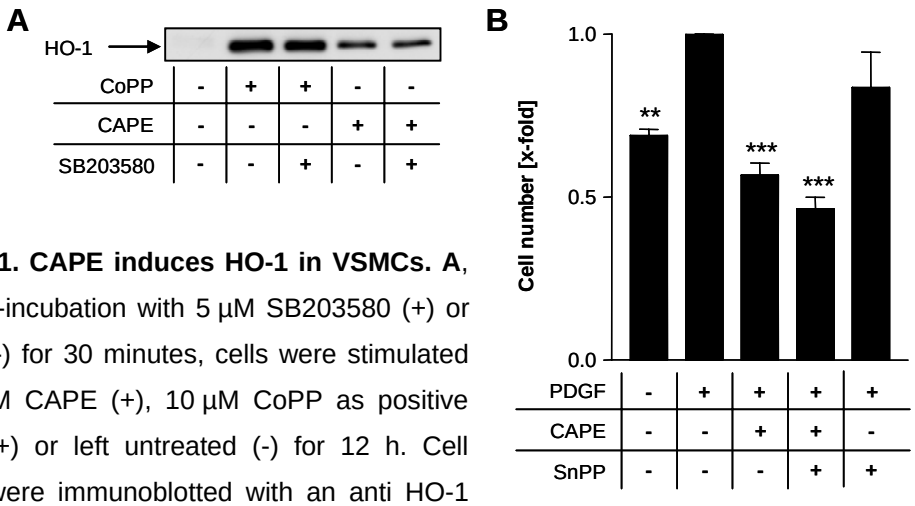


Figure 31. CAPE induces HO-1 in VSMCs. A, After pre-incubation with 5 µM SB203580 (+) or vehicle (-) for 30 minutes, cells were stimulated with 5 µM CAPE (+), 10 µM CoPP as positive control (+) or left untreated (-) for 12 h. Cell lysates were immunoblotted with an anti HO-1 antibody. One representative Western Blot out of three is shown. **B**, **Inhibition of HO-1 does not reduce the growth inhibitory effect of CAPE.** VSMCs were synchronized in G0/G1 as described in materials and methods After pre-treatment with vehicle (-), 5 µM CAPE (+), 10 µM SnPP (+) or with both 5 µM CAPE (+) and 10 µM SnPP (+) for 30 minutes, the cells were stimulated with 20 ng/ml PDGF-BB for 48 hours. The histogram shows the means of cell number ratio of at least three independent experiments, whereas the PDGF values were set as 1. **, $p < 0.01$; ***, $p < 0.001$ (ANOVA/Bonferroni)

2.10 Caveolin-1 is upregulated by CAPE – involvement of p38

Recently, caveolin-1 has been described as a potent regulator of VSM cell signaling pathways, in particular of those resulting in proliferation (Thyberg, 2003; Hassan *et al.*, 2004). Moreover, caveolin-1 expression was shown to occur *via* p38 activation in PDGF-BB-activated VSMC (Kim *et al.*, 2005). We therefore, examined whether CAPE (5 µM) may increase caveolin-1 expression in PDGF-BB-activated VSMCs and whether this effect is reversed by the p38 inhibitor SB203580 (5 µM). Western blot analysis showed that pre-incubation of VSMCs with CAPE before stimulation with PDGF-BB could restore the PDGF-induced degradation of caveolin-1 (figure 32). This effect was neutralized by SB203580. Moreover, anisomycin, an activator of the p38 MAPK pathway, was able to mimic the effect of CAPE.

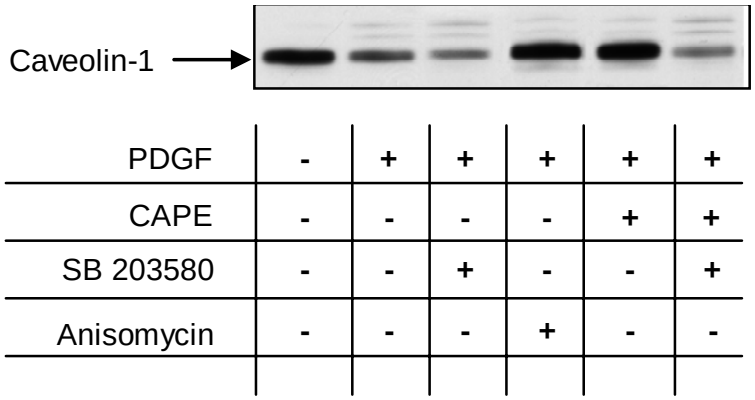


Figure 32. CAPE impairs PDGF-induced caveolin-1 degradation. Cells were pre-incubated with vehicle (-), 5 µM CAPE (+), 5 µM SB203580 or 10 µM anisomycin as positive control for 30 minutes, before stimulation with 20 ng/ml PDGF-BB for 12 h. Cell lysates were immunoblotted with an anti caveolin-1 antibody. One representative Western Blot out of three is shown.

E DISCUSSION

1 Influence of resveratrol on the signaling pathway in VSMC upon Ang II stimulation

The first part of this thesis provides new insight into the putative molecular mechanisms by which RV interferes with Ang II- and EGF-mediated signaling pathways in VSMCs. We were able to show that EGF-R transactivation upon Ang II stimulation is not inhibited by RV. However, RV potently impairs EGF- as well as Ang II-stimulated Akt phosphorylation. The data provided in this work strongly suggest that Shp-2, a protein tyrosine phosphatase previously shown to be involved in EGF-induced PI3K activation in fibroblasts (Wu *et al.*, 2001), is a major part of the involved pathway. Moreover, our data provide evidence that in Ang II- or EGF-activated VSMC Gab1 and Shp-2 play the same important role as they have been shown for EGF signaling to the PI3K/Akt-pathway in fibroblasts.

Ang II has become apparent as a key player in the pathogenesis of vascular diseases. It has been associated with several symptoms of atherosclerosis, such as hypertrophy, proliferation and migration of VSMCs as well as in inflammation and extracellular matrix deposition. This emphasizes its importance for vascular remodeling (Schmidt-Ott *et al.*, 2000). Referring to this, it has recently been shown that transactivation of the EGF-R is important for early Ang II signaling through the AT_1 receptor (Kalmes *et al.*, 2001). Consistent with results reported by others (Ushio-Fukai *et al.*, 2001a), we show a rapid increase in overall EGF-R phosphorylation upon Ang II stimulation. This transactivation was not impaired by RV, indicating that RV acts downstream of the EGF-R. Consistently, Akt phosphorylation is inhibited even when VSMCs are stimulated with EGF instead of Ang II.

PP1, a c-Src inhibitor, is capable of completely inhibiting EGF-induced Akt phosphorylation, and studies performed in other cell systems suggest that c-Src kinase is important for signaling downstream of EGF-R (Sato *et al.*, 1995;

Stover *et al.*, 1995). Interestingly, in HeLa cells, RV has been shown to act as an inhibitor of c-Src tyrosine kinase (Yu *et al.*, 2001). Although c-Src is phosphorylated at various sites, phosphorylation of tyrosine^{418} in the kinase domain is most important, as its autophosphorylation leads to increased kinase activity (Abram and Courtneidge, 2000). Our group found that RV does not alter phosphorylation of c-Src tyrosine^{418} (Haider, 2003). We further found that EGF-induced phosphorylation of tyrosine^{845} of the EGF-R, a highly conserved site previously shown to be phosphorylated in a c-Src dependent manner (Biscardi *et al.*, 1999), is not altered by RV (Haider, 2003). Together with the fact that RV, in contrast to the c-Src inhibitor PP1, does not prevent Ang II-induced EGF-R transactivation, these data show that c-Src activity is not impaired in RV-treated VSMCs.

Most interestingly, RV was not capable of reducing serum-induced Akt phosphorylation, whereas wortmannin, a PI3K-inhibitor that inactivates the catalytic subunit of PI3K, was able to inhibit Akt activation. These observations, strongly suggest that RV acts upstream of PI3K activation when pathways of serum- and Ang II-induced Akt activation have not yet converged. One question concerning growth factor signaling is how specificity is achieved by different stimuli, as many growth factors use the same adapter molecules and downstream effectors. For the EGF-pathway, a positive feedback loop between the adapter Gab1 and PI3K has been proposed, where the PI3K participates both upstream and downstream of Gab1 (Rodrigues *et al.*, 2000). Referring to this, recent work shows that Gab1 and Shp-2 act together in regulating PI3K activity. Briefly, Shp-2 diminishes PI3K activation in response to EGF by specifically dephosphorylating the p85 binding sites on Gab1, thereby interrupting the before mentioned positive feedback loop. Hence, in Shp-2$^{-/-}$ cells, association between Gab1 and p85 is enhanced, leading to an increased activity of PI3K and Akt (Zhang *et al.*, 2002). Most interestingly, this effect has been shown to be specific for EGF since other growth factors such as PDGF or IGF-1 did not lead to enhanced Akt activation in Shp-2$^{-/-}$ cells (Zhang *et al.*, 2002). These results indicate specificity for the EGF-governed pathways. We were able to show that stimulation of VSMCs with EGF as well as with Ang II

leads to a rapid tyrosine-phosphorylation of Gab1 and subsequent association of Gab1 and the p85 subunit of PI3K. Application of RV led to a decreased overall Gab1 phosphorylation, and thus p85 association with Gab1. The inhibition of Gab1 phosphorylation was not as distinct as the inhibition of p85 recruitment to Gab1, especially after stimulation with Ang II. These data correspond to the observation that Shp-2 specifically dephosphorylates only the p85 binding sites on Gab1 (Zhang *et al.*, 2002). Moreover, by confocal microscopy we revealed that EGF-induced translocation of Gab1 and p85 to the plasma membrane is inhibited by RV. Altogether, these results strongly indicate that RV interferes with the association of Gab1 and p85, most likely an early stage in the above-mentioned feedback loop. Our results strongly suggest that the underlying mechanism is inhibition of Gab1 phosphorylation, raising the possibility that Shp-2 may be essential for RV's mechanism of action. In order to clarify the role of Shp-2 in the signaling of RV, we performed experiments using Shp-$2^{-/-}$ fibroblasts and, as a control, Shp-$2^{-/-}$ cells where WT-Shp-2 expression was restored by retroviral gene transduction. As expected, RV did not prevent EGF-induced Akt activation in Shp-$2^{-/-}$ cells, whereas inhibition was highly significant in the reconstituted, WT-Shp-2 expressing control cells. These data suggest that RV may act *via* activation of Shp-2. Further experiments, indicated that RV is capable of activating Shp-2 in RV-treated cells (*in situ)* as well as *in vitro*. Given that the maximal stimulation *in situ* is more dramatic than *in vitro*, it indicates that at least some of the effects of RV are not mediated by direct action of RV on Shp-2. In this context, it is interesting that recent studies have shown that phospho-tyrosine phosphatases (PTPs), including Shp-2 (Meng *et al.*, 2002), are inhibited by peroxide-mediate oxidation of the catalytic cysteine residue (reviewed by (Finkel, 2003). Since previous work has suggested that RV inhibits NADPH oxidase (Nox) enzymes (Orallo *et al.*, 2002), our observations suggest that at least one part of the mechanism of RV may be the inhibition of hydrogen peroxide generation *via* Nox proteins, thus resulting in higher levels of catalytically active Shp-2. Given that Gab1 translocates to the membrane upon EGF stimulation (figure 16) and Shp-2 binds to Gab1, this may bring the phosphatase to the vicinity/proximity of a specific NOX enzyme.

Consistent with our observation that RV only affects Shp-2 activity after EGF stimulation it has been shown that in the absence of stimulation, Shp-2 exists in a closed form in which ROS probably cannot access the active site cysteine (reviewed by (Barford and Neel, 1998). In order to test this model as well as to clarify the mechanism by which RV is capable of directly stimulating recombinant Shp-2, further studies have to be performed.

2 Influence of CAPE on the proliferation in VSMC upon serum- and PDGF-stimulation

In the second part of this study, we show that CAPE abolishes both serum and PDGF-induced VSMC proliferation. The inhibitory concentration (5 µM) upon PDGF-BB stimulation was ten-times below that needed to inhibit NF-κB activity. None of the major classical growth promoting signaling pathways (ERK1/2, PI3K/Akt) was affected by CAPE. In contrast, we found an enhanced and sustained phosphorylation of p38 MAP kinase in response to CAPE contributing to its antiproliferative effect. Our study in association with recent published data (Kim *et al.*, 2005) strongly suggests that CAPE mediates its effect *via* a HO-1-independent activation of p38 MAPK and a subsequent increased expression of caveolin-1 leading to cell cycle arrest at G1 phase.

VSMCs are known to play a crucial role in the development of atherosclerosis and restenosis (Ross, 1999; Newby, 2000). One major autocrine and paracrine mitogen for VSMCs mediating atherosclerotic processes such as hyperplasia, hypertrophy and migration is PDGF (Scapagnini *et al.*, 2002; Yu *et al.*, 2003; Berk, 2001). Maffia *et al.* have recently shown that CAPE is able to reduce restenosis after balloon angioplasty (Maffia *et al.*, 2002). Thus, we hypothesized that CAPE is able to inhibit PDGF-BB-induced VSMC proliferation and sought to identify the mechanisms of this putative effect.

CAPE indeed inhibited PDGF-induced VSMC growth dose-dependently (1-5 µM). Interestingly, this effect was associated with an increased and prolonged phosphorylation of p38 MAPK. The role of p38 MAPK in cell proliferation is controversially discussed: activation of p38 MAPK was shown to result in cell proliferation in VSMC after vascular injury (Ju *et al.*, 2002). On the other hand, some data show that activation of p38 MAPK correlates with inhibition of cell cycle progression at G1/S transition (Molnar *et al.*, 1997) and that inhibition of p38 MAPK stimulates proliferation (Philips *et al.*, 2000). These findings indicate that p38 MAPK is able to differentially regulate cell proliferation depending on cell type, stimulus and cell cycle position (Ambrosino and

Nebreda, 2001). Prolonged p38 phosphorylation in response to CAPE was not only associated with VSM cell cycle arrest but also inhibited by the p38 inhibitor SB203580 suggesting that p38 contributes to the growth inhibitory effect of CAPE.

CAPE has been described as a potent NF-κB inhibitor (Natarajan *et al.*, 1996) and NF-κB is shown to mediate cell proliferation and cell cycle progression in various cell types (Mehrhof *et al.*, 2005). Activation of NF-κB in response to PDGF-BB in VSMC, however, is disputed (Mehrhof *et al.*, 2005; Peppel *et al.*, 2005; Son *et al.*, 2006) We, therefore, decided to monitor NF-κB binding and transcriptional activity in response to CAPE in order to see whether NF-κB could mediate CAPE-induced cell cycle arrest. In agreement with our results data provided by (Natarajan *et al.*, 1996) showed that CAPE acts as NF-κB inhibitor only at concentrations higher than 50 µM. Thus, we exclude the possibility that CAPE mediates its antiproliferative effect by inhibition of NF-κB.

Heme oxygenase-1 (HO-1) plays a central role in regulating intracellular heme levels by catalyzing the first and rate-limiting step in the degradation of heme (Tenhunen *et al.*, 1968) thereby generating carbon monoxide (CO), biliverdin and free iron. Inhibition of HO-1 by zinc or tin protoporphyrin IX potentiates the mitogenic action of serum, endothelin, angiotensin II and PDGF in VSMCs (Choi *et al.*, 2004; Peyton *et al.*, 2002; Durante, 2002; Togane *et al.*, 2000) pointing to an antiproliferative effect of HO-1. Further evidence was given by Duckers *et al.* using VSMC from HO-1 knockout mice. These cells exhibited enhanced DNA synthesis and proliferation compared to wild type SMCs (Duckers *et al.*, 2001). Thus, substantial evidence indicates that HO-1 is a negative regulator of growth in VSMCs by means of CO (Durante, 2002; Peyton *et al.*, 2002). In addition, CAPE is a potent inducer of HO-1 in astrocytes (Scapagnini *et al.*, 2002).The antiproliferative effect of CAPE could, therefore, also be mediated by its ability to induce HO-1 (Scapagnini *et al.*, 2002). Although we were able to confirm induction of HO-1 by CAPE in VSMCs, the antiproliferative effect of CAPE was not affected by HO-1 inhibition suggesting that CAPE-induced cell cycle arrest occurs independently of HO-1 induction.

Caveolin-1 was discovered in the early 1990s and is a major structural component of caveolae, invaginations of the cell membrane contributing to the regulation of many cellular processes such as eNOS activity, EGF-R or PDGF-R signaling (Schlegel *et al.*, 1998). The role of caveolin-1 in VSMCs has not yet fully been characterized, but studies performed by Peterson *et al.* propose an antiproliferative role for this membrane protein, especially upon stimulation with PDGF (Peterson *et al.*, 1999; Peterson *et al.*, 2003). In agreement with these data a recent study has shown that caveolin-1 negatively regulates VSMC proliferation and neointima formation of injured aorta (Hassan *et al.*, 2004). Moreover, a recently published study has established a clear link between CO, p38 MAPK activation and caveolin-1 up regulation. Herein, they show that CO caused p38 phosphorylation which in turn resulted in increased caveolin-1 level leading to VSMC growth arrest (Kim *et al.*, 2005). In agreement with these data, CAPE led to a strong rise of caveolin-1 in the presence of PDGF compared to PDGF alone and the specific p38 inhibitor SB203580 was able to reverse this effect. In contrast to published data by Kim *et al.* (2005), however, activation of p38 MAPK did not occur *via* the HO-1/CO pathway. Since the second messenger of CO is cGMP it is conceivable that CAPE increases intracellular cGMP levels in VSMC similarly as demonstrated earlier for red wine polyphenols (El-Mowafy, 2002; Dell'Agli *et al.*, 2005). Further experiments will be necessary to clarify a possible role of cGMP in CAPE signaling.

F SUMMARY

1 Effects of resveratrol on the signaling pathway in VSMC upon Ang II stimulation

In this thesis we provide important new insight into the mechanisms by which RV interacts with Ang II- and EGF-induced signaling pathways involved in the pathogenesis of cardiovascular disease. One of these pathways is the PI3K/Akt pathway which was investigated more detailed. We were able to show that although EGF-R transactivation is important for Akt activation, RV does not interfere with this step. Additional experiments revealed that the most probable target of RV lies downstream of EGF-R but upstream of Akt activation. Refering to this, it is important to note that we showed for the first time an important and specific role for Shp-2 and Gab1 in EGF signaling to the PI3K/Akt pathway in VSMCs. Furthermore, we were able to demonstrate that RV decreased binding of Gab1 to the PI3K subunit p85 and subsequent translocation of the complex to the plasma membrane. Most importantly, we were able to show that Gab1 phosphorylation and p85 recruitment to Gab1 was also decreased upon Ang II stimulation. By continuative analysis we revealed that Shp-2 is responsible for the Gab1 dephosphorlyation and that RV seems to be capable of activating Shp-2. As this thesis provides new details in early signaling events triggered by EGF and Ang II regarding the role of Shp-2 and Gab1, it assists to elucidate the molecular mechanisms elicited by RV in VSMCs. Therefore, it may be helpful to analyze the suitability of RV as a putative therapeutic or preventive agent in cardiovascular disease.

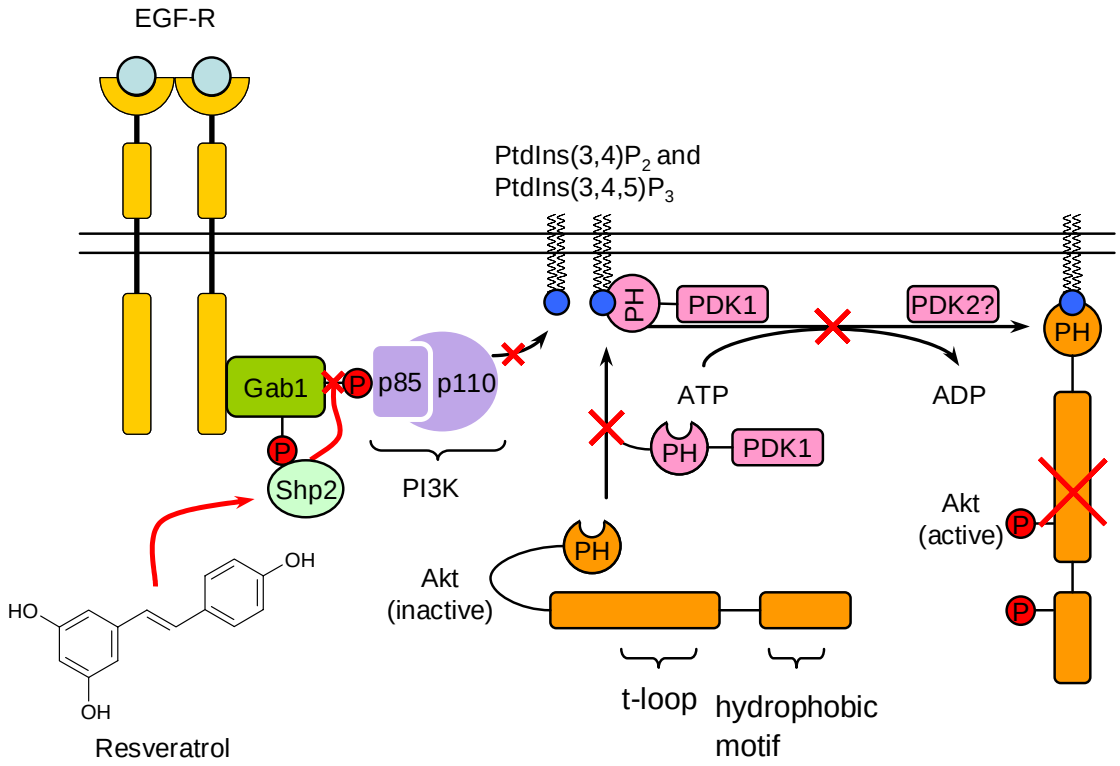


Figure 33. Resveratrol acts *via* the phosphatase Shp-2. The exact target of resveratrol is not yet identified. However, Shp-2 is the most upstream target shown to be involved in the depicted EGF-R-mediated signaling cascade.

2 Effects of CAPE on the proliferation of VSMC upon serum- and PDGF-stimulation

In the present work, we have clearly demonstrated that CAPE inhibits both, serum and PDGF-induced VSMC proliferation. Effective concentrations upon PDGF-BB stimulation were as low as 5 µM. These properties are most likely mediated by p38 MAPK phosphorylation and subsequent upregulation of caveolin-1. Our study provides first insights into the mechanism by which CAPE interferes with signaling cascades initiated upon stimulation of VSMC with PDGF-BB. It is noteworthy that we provided evidence that HO-1 does not contribute to the anti-proliferative property of CAPE whereas caveolin-1 is up regulated in a p38-dependant manner. Consistent with previous studies (Peterson *et al.*, 2003; Kim *et al.*, 2005; Peterson *et al.*, 1999), caveolin-1 appears to be important in proliferative signaling in VMSC, and may, therefore,

be considered as a target for new therapeutic approaches to overcome neointimal remodeling and restenosis.

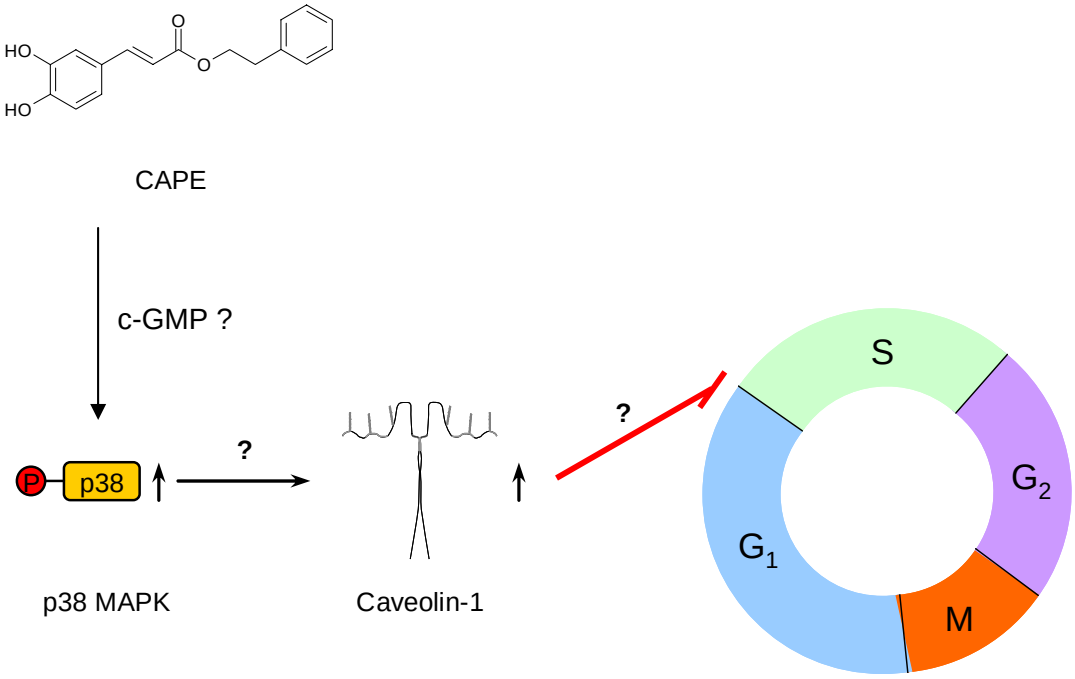


Figure 34. CAPE inhibits VSMC proliferation *via* a HO-1-independent activation of p38 MAPK and a subsequent increased expression of caveolin-1 in a p38 MAPK-dependent manner.

G APPENDIX

1 Abbreviations

7-AAD	7-amino-actinomycin D
ACE	angiotensin-converting enzyme
AG	AG 1478
Ang II	angiotensin II
AP-1	activating protein 1
APS	ammonium persulfate
Aph	aphidicolin
$AT_{1/2}$-R	angiotensin II type 1/2 receptor
bFGF	basic fibroblast growth factor
BrdU	bromodeoxyuridine
BSA	bovine serum albumine
C	Celsius
CAPE	caffeic acid phenethyl ester
Cav-1	caveolin-1
CBP	CREB binding protein
Cdk	cyclin-dependent kinase
Co	control
CREB	cAMP-response-element binding protein
CS	calf serum
DNA	deoxyribonucleic acid
DAG	diacylglycerol
DMEM	Dulbecco’s Modified Eagle’s medium
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
EGTA	ethylene glycol–bis(2-aminoethyl ether) tetraacetic acid

eIF2B	eukaryotic initiation factor 2B
Erk 1/2	extracellular-signal regulated kinase 1/2
FOXO	FOXO family of Forkhead transcription factors
GPCR	G-protein coupled receptor
Gab 1	Grb 2-associated binder 1
Grb 2	growth factor receptor bound protein 2
GSK 3	glycogen synthase kinase 3
Gy	Gray
h	hour
HB	heparin-binding
IL 6	interleukin 6
IP_3	inositol trisphosphate
JNK	c-Jun N-terminal kinase
LDL	low density lipoprotein
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein 1
m	milli
M	molar
Mdm2	mouse double minute 2
min	minutes
MKK (MEK)	MAPK kinase
MKKK	MAPK kinase kinase
mTor	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	messenger RNA
n	nano
NAC	N-Acetyl-L-Cysteine
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NFκB	nuclear factor κB
NO	nitric oxide
NOS	nitric oxide synthase

oxLDL	oxidised LDL
p	phospho
PAA	polyacrylamide
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PDK 1	3-phosphoinositide-dependent kinase I
PH	pleckstrin homology
PI	propidium iodide
PI3-k	phosphoinositide-3 kinase
PKA/B/C/G	protein kinase A/B/C/G
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
Poly[dIdC]	Polydesoxyinosine-desoxycytosine
Ppm	parts per million
PP2A	protein phosphatase 2A
PtdIns	phosphatidylinsositol
PTEN	phosphatase and tensin homologue deleted from chromosome 10
PYK 2	proline rich tyrosine kinase 2
R	restriction point
Rb	Retinoblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RV	resveratrol
S, Ser	serine
SDS	sodium dodecyl sulphate
SEM	Standard error of the mean value
SH1/2	Src homology 1/2
Shc	Src-homology 2 domain containing
SHIP	Src-homology 2-containing inositol phosphatase

Shp-2	Src-homology 2-containing phosphatase-2
Sos	son of sevenless
T, Thr	threonine
TAE	Tris, acetate, EDTA buffer
TBE	Tris, borate, EDTA buffer with Triton X-100
TBS-T	Tris buffered saline solution with Tween
TEMED	N,N,N´,N´-tetramethylethylenediamine
TGF-β	transforming growth factor β
TRIS	Tris(hydroxymethyl)-aminomethane
v	volume
VSMC	vascular smooth muscle cell
w	weight
WM	wortmannin
Y, Tyr	tyrosine
µ	micro

2 Alphabetical order of companies

Amersham Pharmacia	Freiburg, Germany
Beckman Instruments	Munich, Germany
BD biosciences	Heidelberg, Germany
Biochrom	Berlin, Germany
BioRad Laboratories	Munich, Germany
BioSource	Nivelle, Belgium
Calbiochem	Schwalbach, Germany
Charles River GmbH	Sulzfeld, Germany
Cell Signaling	Frankfurt, Germany
Eppendorf	Maintal, Germany

Invitrogen	Karlsruhe, Germany
Jackson ImmunoResearch	West Grove, USA
Millipore	Eschborn, Germany
NEN	Cologne, Germany
Pan Biotech	Aidenbach, Germany
Qbiogene-Alexis	Grünberg, Germany
PAA Laboratories	Linz, Austria
PAN	Aidenbach, Germany
Peske	Aindling-Pichl, Germany
PharMingen	San Diego, CA, USA
Promega	Heidelberg, Germany
Roche	Mannheim, Germany
Roth	Karlsruhe, Germany
Serotec	Eching, Germany
Sigma-Aldrich	Taufkirchen, Germany
Shimadzu	Duisburg, Germany
Tecan	Crailsheim, Germany
Tocris	Ellisville, USA
Upstate Biotechnology	Lake Placid, USA
Zeiss	Oberkochen, Germany

3 Publications

3.1 Abstracts

T.U. Roos, U.G.B. Haider, A.M. Vollmar, V.M. Dirsch.

Resveratrol impairs angiotensin II-mediated Akt activation in vascular smooth muscle cells by affecting the phosphotyrosine phosphatase Shp-2.

Naunyn Schmiedebergs Arch Pharmacol. 2005 Feb;371(Supp. 1):R15

3.2 Original publications

Haider UG, Roos TU, Kontaridis MI, Neel BG, Sorescu D, Griendling KK, Vollmar AM, Dirsch VM.

Resveratrol inhibits angiotensin II- and epidermal growth factor-mediated Akt activation: role of Gab1 and Shp-2

Mol Pharmacol. 2005 Jul;68(1):41-8

Thomas U. Roos, Andrea Schwaiberger, Angelika M. Vollmar, Verena M. Dirsch.

p38 mitogen-activated protein kinase-mediated induction of caveolin-1 contributes to the antiproliferative effect of caffeic acid phenethyl ester in rat aortic vascular smooth muscle cells

Manuscript in preparation

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Ref Type: Thesis/Dissertation

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I ACKNOWLEDGEMENTS

This work was established from September 2002 to January 2006 at the LMU Munich, Center of Drug research, Department of Pharmacy, Pharmaceutical Biology, in the laboratories of Mrs. Prof. Dr. A. M. Vollmar.

First and foremost, I would like to thank Prof. Dr. Angelika M. Vollmar for giving me the opportunity to achieve this doctoral thesis work in her laboratory. I am deeply grateful for her support, encouragement, expert guidance and confidence.

I am also very grateful to my supervisor, Prof. Dr. Verena M. Dirsch, for her constant support, encouragement and for always being open for questions. Her excellent expert guidance and interesting discussions contributed to a comfortable atmosphere and were very supportive.

Special thanks go to the thesis committee, especially to PD Dr. Erl for acting as second examiner.

Many thanks go to PD Dr. Stefan Zahler for his helpful hints and tricks.

Furthermore, I am appreciative to Dr. Rainer Samtleben, for his useful suggestions and motivating discussions.

I am much obliged to my friends and colleagues in the lab for listening, just having fun and always being there, whenever I needed, especially to Anita, Anja, Lila, Rita, and all the others. Thank you Thommäß for your friendship, the "Feierabendbier", the activities in Mainz and outside the lab, and support in all circumstances. Thanks go also to Hanna for her help. Very special thanks to my first lab-mate, Dr. Irina Müller, for cheering me up every time and taking the time for proofreading my dissertation. I am deeply thankful to my second "Boxenluder" Dr. Nancy Lopez, for her cheerful character, her great support during the creation of this work, and the untiring attempts to teach me tidiness. I am much obliged to Dr. Guido Jürgenliemk for his close friendship, his amusing stories, and, of course, his support during a hard time. I also want to thank Uta Jürgenliemk for her acceptance.

Last, but not least I want to express my deepest gratitude to my family for their outstanding encouragement and understanding and to Miriam for her sympathy, and exceptionally promoting me during the compilation of this work.

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