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Resveratrol Attenuates Vascular Smooth Muscle Cell Hypertrophy and Hyperplasia:

Elucidation of Signalling Pathways

von

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Erklärung

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B. Introduction

1 Background and aim of the work

Cardiovascular disease is currently the leading cause of death in the developed countries, and atherosclerosis is by far the most prominent contributor (Murray and Lopez, 1997; Libby, 2002). Considerable effort is therefore made to develop new therapeutic strategies to combat and reduce cardiovascular complications. One possibility is to investigate natural products, especially those that are, thanks to epidemiological studies or their use in folk medicine, already discussed to have potentially beneficial effects in this field.

In this regard, the polyphenolic stilbene-derivate *trans*-resveratrol (*trans*-3,5,4`-trihydroxystilbene) was a good candidate. As a major constituent of red wine, this substance has been proposed to partially account for the beneficial effects attributed to this beverage in cardiovascular disease (Wu *et al.*, 2001; Soleas *et al.*, 2001).

Vascular smooth muscle cells (VSMCs) are the prime cellular component of the normal artery as well as of intimal lesions that develop in response to arterial injury. Consequently, hypertrophy and hyperplasia of VSMCs are hallmarks of vascular disorders such as atherosclerosis and restenosis (Touyz and Schiffrin, 2000; Lusis, 2000; Dzau *et al.*, 2002).

The purpose of this study was therefore to investigate the effects of resveratrol on VSMC hypertrophy and hyperplasia. To achieve this aim we employed primary rat aortic smooth muscle cells, as these are widely used and accepted in cardiovascular research. Angiotensin II (Ang II), the main effector peptide of the renin-angiotensin system, was applied to induce VSMC hypertrophy, while calf serum, a classical mitogenic stimulus, was used for induction of cellular proliferation.

In particular, the following questions were addressed:

1. Is resveratrol able to attenuate VSMC hypertrophy induced by Ang II-treatment, and what are the mechanisms underlying a putative effect?
2. What signalling pathways are involved in mediating the antiproliferative properties exerted by resveratrol in calf serum-treated VSMCs?

2 Vascular Smooth Muscle Cells

2.1 VSMCs in neointima formation and atherosclerosis

In intact arteries, VSMCs are normally in a quiescent, non-proliferative state. However, upon stimulation by mechanical injury or growth factors, VSMCs enter the cell cycle and start to replicate. After carotid injury, VSMC proliferation begins within hours, and by day 4 cells start to migrate to the intima, where proliferation continues for approximately 2 weeks. Finally, extracellular matrix (ECM) synthesis starts and additionally increases intimal volume (Braun-Dullaeus *et al.*, 1998). At first, thickening of the atherial wall is compensated by gradual dilation, a process referred to as vascular remodelling, but continuous increase in intimal mass finally hampers blood flow and results in clinical manifestation (Libby, 2002; Ross, 1999).

2.2 VSMCs in culture

Although cultured VSMCs retain many characteristics of their in vivo counterparts, culturing leads to changes in expression of some smooth muscle specific proteins and properties, for example loss of the cyclic GMP-dependent kinase (PKG) (Lincoln *et al.*, 2001; Brophy *et al.*, 2002). Concomitantly, VSMCs adopt a non-contractile, more synthetic phenotype characterised by enhanced proliferation and extracellular matrix deposition that resembles intimal VSMCs (Shanahan and Weissberg, 1998). Therefore, subcultured VSMCs are a suitable model for cells found in the neointima of atherosclerotic leasons.

3 Resveratrol

3.1 Occurrence and history

Resveratrol (*trans*-3,5,4´-trihydroxystilbene) is found in a narrow range of spermatophytes, including grapes, peanuts and mulberries. It was first isolated 1963 from the roots of the weed *Polygonum cuspidatum* that has a long tradition in Chinese and Japanese folk-medicine, but major interest has not been focused on this compound until its discovery in red wine 1992 (Soleas *et al.*, 1997). The highest concentrations of resveratrol are found in grape skin (50-100 µg/g), and due to its physiological role as a phytoalexin it is more abundant in grapes grown in cooler climates where fungal infections are more common.

3.2 Biosynthesis and biological function

Resveratrol is synthesised from phenylalanine in several steps (Fig. 1). Phenylalanine derived from the Shikimate pathway is converted to cinnamic

acid through oxidative deamination by the action of phenylalanine ammonia lyase. Cinnamic acid is enzymatically hydroxylated to *p*-coumaric acid, which is subsequently converted to the CoA ester derivate by a specific CoA ligase. Condensation of 4-coumaroyl CoA with three malonyl CoA units by the enzyme resveratrol synthase, a stilbene synthase, results in the formation of resveratrol (Wu *et al.*, 2001).

The main task of resveratrol and its condensation products, a family of polymers given the name viniferin, in the plant cell is to function as a phytoalexin. Consequently, amounts are greatly increased upon fungal infection especially in the skin, and barely detectable in the flesh of healthy grapes. These findings implicate that resveratrol synthase is not expressed constitutively but induced upon UV-irradiation, trauma and infection. In contrast, chalcone synthase, a constitutively active enzyme, is able to catalyse the generation of a chalcone from *p*-coumaroyl CoA and three malonyl CoA in a pathway alternative to stilbene synthesis. Chalcones are then further converted to flavonoids (Soleas *et al.*, 2001).

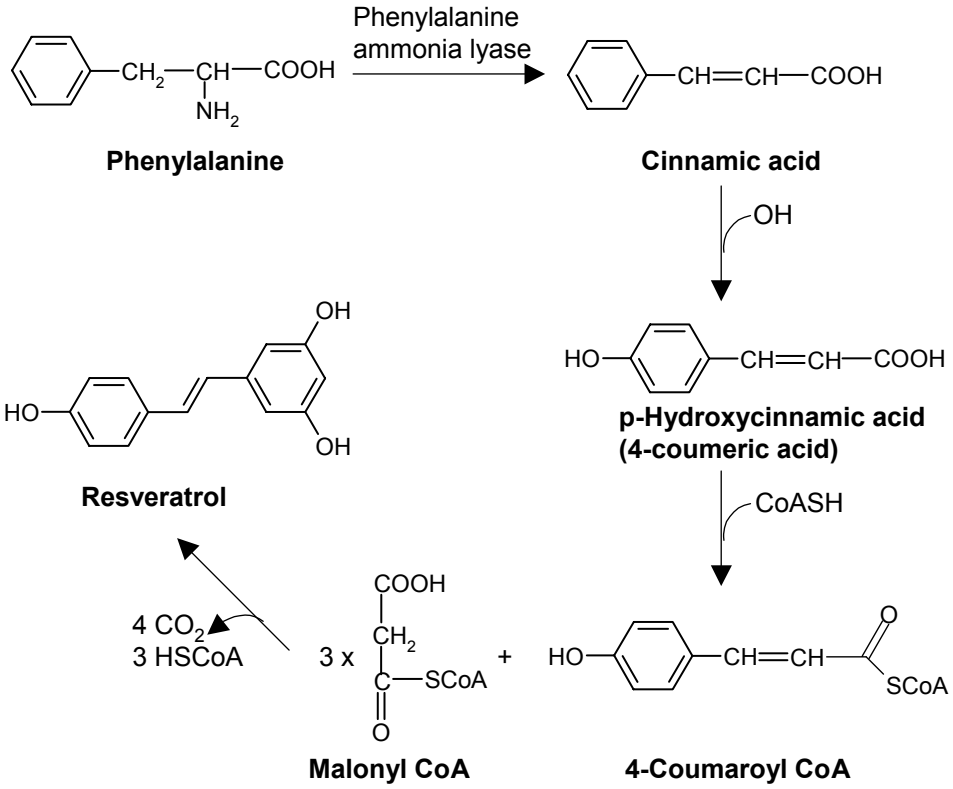

Fig. 1. Biosynthesis of resveratrol

3.3 Properties

Since its discovery in red wine 1992 (Siemann EH and Creasy LL, 1992), a plethora of studies have been performed, generating a vast and still increasing number of biological properties attributed to resveratrol. In addition, many of these effects seem to be tissue- as well as cell type specific (for review, see (Wu *et al.*, 2001; Soleas *et al.*, 2001; Gusman *et al.*, 2001). For that reason, this paragraph will focus on distinct features of major interest for this substance.

3.3.1 Antioxidant activity

Resveratrol has proved to be a potent antioxidant in many biological systems (Soleas *et al.*, 2001). Its antioxidant properties have been demonstrated for the first time by Frankel *et al*. (Frankel *et al.*, 1993), revealing that resveratrol was more potent than α-tocopherol but less potent than quercetin in inhibiting copper-catalysed oxidation of human low density lipoprotein (LDL) *in vitro*.

3.3.2 Oestrogenic activity

Resveratrol has structural similarities with diethylstilbestrol, a synthetic oestrogen, and Gehm *et al*. were the first to describe binding of resveratrol to the oestrogen receptor and transcription of oestrogen-responsive reporter genes transfected into human breast cancer cells. However, the extent of these effects was different according to the cell type investigated (Gehm *et al.*, 1997), and so far, *in vivo* studies have failed to show oestrogenic activities in whole animals (Soleas *et al.*, 2001).

3.3.3 Chemopreventive and anti-inflammatory activity

Resveratrol has been demonstrated to interfere with cyclooxygenases (COX), but alterations of COX gene expression and activity by resveratrol are variable in different experimental models (Jang *et al.*, 1997; Subbaramaiah *et al.*, 1998). However, anti-inflammatory properties of resveratrol were confirmed *in vivo*, as resveratrol strongly suppressed carrageenan-induced paw oedema in both the acute and the chronic phase (Jang *et al.*, 1997). In addition to interference with COX, inhibition of the 5-lipoxygenase and the 15-lipoxygenase pathway was demonstrated in several models (Soleas *et al.*, 2001).

Most studies agree that resveratrol is able to induce apoptosis in many cancer cell lines *via* p53-dependent as well as p53-independent pathways (Lin *et al.*, 2002; She *et al.*, 2001; Tinhofer *et al.*, 2001; Mahyar-Roemer *et al.*, 2001). Nevertheless, one report describes inhibition of hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells by resveratrol (Jang and Surh, 2001).

Furthermore, resveratrol was shown to inhibit activation of two important transcription factors, activating protein 1 (AP-1) and nuclear factor κB (NF-κB). The former experiments were conducted in PMA- and UV-irradiated Hela cells. The authors suggested inhibition of protein kinase C (PKC) and tyrosine kinases to be responsible for this effect (Yu *et al*.,2001). Suppression of NF-κB activation has been linked to decreased IκB-degradation that seemed to be, in turn, caused through interference with yet unidentified *upstream* pathways (Holmes-McNary and Baldwin, Jr., 2000; Tsai *et al.*, 1999).

Additionally, in Hep G2 cells, resveratrol inhibits induction of mRNA, protein expression and enzyme activity of the cytochrome P_{450}-dependent enzyme CYP1A1 that is involved in dioxin metabolism (Ciolino *et al.*, 1998; Casper *et al.*, 1999), although, on the other hand, others report induction of CYP1A1 mRNA by resveratrol in HeLa cells (Frotschl *et al.*, 1998).

Another aspect of resveratrol`s putative anticarcinogenic activities is its ability to induce quinone reductase, a phase II enzyme capable of metabolically detoxifying carcinogens (Jang *et al.*, 1997).

3.3.4 Influence on cell cycle and proliferation

Resveratrol exerts antimitogenic properties in a number of cell lines including VSMCs, inhibiting proliferation and DNA synthesis in a dose-dependent manner (Zou *et al.*, 2000; Zou *et al.*, 1999; Adhami *et al.*, 2001; Sgambato *et al.*, 2001; Wolter *et al.*, 2001). In this regard, cell free *in vitro* experiments revealed that resveratrol is an inhibitor of ribonucleotidreductase (Fontecave *et al.*, 1998) as well as DNA-polymerase α and δ (Stivala *et al.*, 2001). Depending on the cell type investigated, resveratrol showed divergent effects on other important cell cycle proteins like the cyclins, the retinoblastoma protein (Rb) or $p21^{Cip1}$ (Wolter *et al.*, 2001; Adhami *et al.*, 2001; Ahmad *et al.*, 2001; Hsieh *et al.*, 1999; Hsieh and Wu, 1999; Ragione *et al.*, 1998; Kawada *et al.*, 1998). In VSMCs, however, modulation of these proteins by resveratrol has never been investigated.

3.3.5 Cardiovascular effects

In addition to antiproliferative and antioxidative effects, prevention of platelet-aggregation by resveratrol has been described in several studies (Pace-Asciak *et al.*, 1995; Pace-Asciak *et al.*, 1996; Bertelli *et al.*, 1995).

Furthermore, resveratrol has been shown to reduce tumour necrose factor α (TNF-α)-induced expression of the adhesion molecules ICAM-1 and VCAM-1 as well as adhesion of U937 monocytoid cells to human saphenous vein endothelial cells (Ferrero *et al.*, 1998).

Moreover, Zou *et al.* provided evidence that resveratrol is capable of reducing intimal hyperplasia of the injured vascular wall in a rabbit model (Zou *et al.*, 2000).

More recently, Orallo *et al.* reported that resveratrol increased NO bioavailability *via* inhibition of NADPH oxidase activity in rat aortic homogenates, while eNOS activity was not altered under these conditions (Orallo *et al.*, 2002). Regarding eNOS activity, similar results were obtained in our group (Leikert *et al.*, 2002), but others report an increase in eNOS activity, protein and mRNA by resveratrol (Wallerath *et al.*, 2002).

3.3.6 Bioavailability

Recent attention has been drawn to bioavailability of resveratrol, allowing better evaluation of the likeliness of *in vivo* efficacy. Goldberg *et al.* reported that Resveratrol was readily absorbed with an assumed bioavailability around 90%; however, clearance from the blood stream was very rapid (Soleas *et al.*, 2001). On the other hand, Bertelli *et al*. suggested an accumulation of resveratrol in different organs after prolonged administration to rats (Bertelli *et al.*, 1996), while Kuhnle *et al*. investigated absorption and metabolism of resveratrol in an isolated small intestine model and found that the majority of resveratrol was absorbed as a glucuronide conjugate (Kuhnle *et al.*, 2000). This has been

confirmed by a recent study showing that *trans*-resveratrol-3-O-glucuronide and *trans*-resveratrol-3-sulfate were the prime metabolites found in rat urine and mouse serum (Yu *et al.*, 2002).

So far, too little is known for judging the amounts of resveratrol needed in humans to obtain – potentially - beneficial effects. However, evaluating the existing data on resveratrol`s bioavailability, it seems unlikely that biologically active amounts of resveratrol can be reached exclusively by means of ingestion of resveratrol-containing foods.

4 Angiotensin II

4.1 History

Synthesis and pharmacology of Ang II were first described more than 50 years ago. Since then, Ang II was seen primarily as a potent and important vasoconstrictor. It was not until the beginning of the 1990s that Ang II was implicated in vascular pro-inflammatory and proliferative actions. Subsequently, however, rapid progress was made in understanding the mechanistic background of these findings (Alexander and Dzau, 2000).

4.2 Structure and biosynthesis

Ang II is an octapeptide hormone that can be produced systemically *via* the circulating (also termed renal) renin-angiotensin system, or locally by means of tissue renin-angiotensin systems (Touyz and Schiffrin, 2000).

In the circulating renin-angiotensin system, renal-derived renin cleaves the hepatic-derived α-globulin angiotensinogen at the N-terminus, thus producing Ang I, a decapeptide. In the lungs, Ang I is converted into Ang II by the action of the dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE).

This step can be catalysed also by several non-ACEs, such as chymase, carboxypeptidase and cathepsin G. However, although for example chymase is present in atherosclerotic lesions, Ang II colocalises with ACE rather than with chymase, suggesting that Ang II generation in the atherosclerotic vessel depends predominantly on ACE (Schmidt-Ott *et al.*, 2000).

In addition, Ang I can be transformed to the heptapetide Ang II (1-7) by tissue endopeptidases. Ang II (1-7) seems to be a naturally occurring antagonist of Ang II actions, with vasodepressor as well as antihypertensive properties (Touyz and Schiffrin, 2000).

Degradation of Ang II to Ang III and Ang IV is performed by aminopeptidases (Touyz and Schiffrin, 2000).

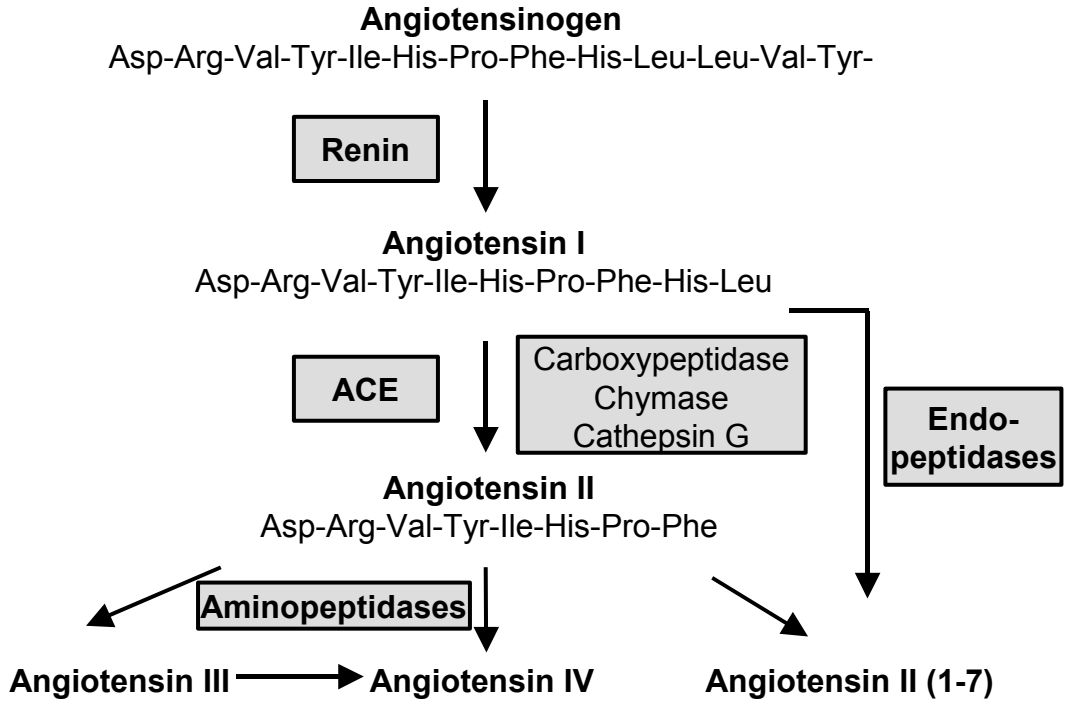

Fig. 2. Pattern of the renin-angiotensin system. Adapted from (Touyz and Schiffrin, 2000).

In recent years, it has become evident that generation of Ang II is not limited to the circulating renin-angiotensin system; instead, all components are expressed in the vessel wall (Schmidt-Ott *et al.*, 2000).

Angiotensinogen-mRNA was found in VSMCs of healthy blood vessels, and levels increased in the media as well as in the neointima after aortic balloon injury in rats (Rakugi *et al.*, 1993). Renin mRNA and protein were detected predominantly in the media and again, elevated levels were observed 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). Interestingly, a recent study showed that in the human forearm in situ generated Ang II is more important for vasoconstriction than circulating Ang II (Saris *et al.*, 2000).

Taken together, there is evidence that these tissue renin angiotensin systems may play an important role in local regulation of blood flow as well as in the pathogenesis of cardiovascular disease (Touyz and Schiffrin, 2000; Weiss *et al.*, 2001).

4.3 Receptors

Ang II mediates its effects through the action of the G protein-coupled receptors (GPCRs) AT_1 and AT_2. AT_3 and AT_4 receptors have been identified, but their pharmacology is not entirely clear yet (Touyz and Schiffrin, 2000).

Most of the vascular effects of Ang II are mediated by the AT_1-R, a glycoprotein composed of 359 amino acids that activates phospholipase C (PLC) *via* a heterotrimeric G_q protein (Touyz and Schiffrin, 2000).

In the vessel wall, AT_1-R are localised mainly in VSMCs. Levels in the adventitia are low, whereas endothelial cells do not seem to contain significant amounts of AT_1-R (Allen *et al.*, 2000; Zhuo *et al.*, 1998). Of interest, the density of AT_1-R is increased in the media of diseased blood vessels compared to healthy animals (Yang *et al.*, 1998). While, in rodents, two subtypes termed AT_{1A} and AT_{1B} sharing 94% sequence homology have been characterised, there is only one AT_1-R in humans (Allen *et al.*, 2000).

The AT_2-R is, as well, a seven-transmembrane GPCR, but has low sequence homology (around 32%) with the AT_1-R. It is a 363 amino acid protein expressed at high levels in foetal tissues and decreases rapidly after birth (Yamada *et al.*, 1999). In healthy blood vessels, only 10% of the total angiotensin receptors are of the AT_2-type (Schmidt-Ott *et al.*, 2000). AT_2 seem to be localised predominantly in adventitia and endothelial cells, but not in VSMCs (Wang *et al.*, 1998; Zhuo *et al.*, 1998). There is evidence that AT_2-R levels, in contrast to AT_1-R, are not altered in atherosclerosis (Yang *et al.*, 1998).

So far, the main function of the AT_2-R is believed to lie in functionally antagonising the vasoconstrictor actions of the AT_1-R. This is achieved by inhibition of the proliferative and growth-promoting effects mediated by the AT_1-R and by inducing apoptosis (Allen *et al.*, 2000).

4.4 Role in haemodynamics

Ang II leads to VSMC contraction within seconds. This effect is a consequence of PLC activation through the G_q-coupled AT_1-R and subsequent increase in intracellular Ca^{2+} levels (Touyz and Schiffrin, 2000). Ca^{2+} associates with Calmodulin, leading to activation of the myosin light chain kinase that is a prerequisite for contraction (Morano, 1992).

Ca^{2+}-increase after Ang II stimulation is biphasic. PLC hydrolyses 4,5-phosphorylated phosphatidylinositol (PtdIns) to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilises intracellular Ca^{2+} from the sarcoplasmatic reticulum, leading to a rapid transient increase in Ca^{2+} levels. The second wave is more sustained and results from transmembrane Ca^{2+} influx. The exact signalling mechanisms triggered by Ang II to induce Ca^{2+} influx are unclear, but direct or indirect activation of different Ca^{2+} channels have been proposed. In addition to IP_3-mediated mobilisation of intracellular Ca^{2+} and influx of extracellular Ca^{2+}, tyrosine kinase-dependent increase in intracellular Ca^{2+} has been suggested (Touyz and Schiffrin, 2000).

The other second messenger generated by PLC, DAG, also contributes to vasoconstriction. DAG, together with Ca^{2+} and phosphatidylserine, activates PKC, a serine/threonine kinase existing in at least 11 isoforms. PKC stimulates activation of the Na^+/H^+ exchanger, leading thus to intracellular alkalinisation. It has been demonstrated that activation of the Na^+/H^+ exchanger and alkalinisation induces vasoconstriction by increasing Na^+ and Ca^{2+} and sensitising the contractile machinery (Touyz and Schiffrin, 2000).

Additionally, Ang II regulates blood pressure and plasma volume by stimulation of renal Na^+ and water resorption, aldosterone release, sympathetic nervous activity and thirst responses (Touyz and Schiffrin, 2000).

4.5 Role in development of cardiovascular disease

Promotion of cardiovascular disease by Ang II is not limited to VSMCs but involves all components of the vessel wall. In this chapter, a short summary of Ang II actions in the vascular wall in general will be given first. Subsequenty, the role of Ang II in VSMC responses will be discussed more detailed.

4.5.1 Ang II actions in the vessel wall

Atherosclerosis is now considered to be a chronic inflammatory disease, initiated by accumulation of lipid-laden macrophages and endothelial dysfunction (Ross, 1999). Interestingly, Ang II can mimic many features of atherosclerosis, and triggers both inflammation and oxidative stress (Weiss *et al.*, 2001).

Ang II is critically involved in generation of reactive oxygen species (ROS) in the vessel wall. The most important source of ROS in the vessel is NAD(P)H oxidase, an enzyme first discovered in leukocytes but now known to be expressed also in vascular cells. Other potential sources of ROS are xanthine oxidase, lipoxygenases, cytochrom P_{450} monooxigenases and myeloperoxidase (Griendling *et al.*, 1994; Zhang *et al.*, 1999; Warnholtz *et al.*, 1999; Hayashi *et al.*, 2001).

Enhanced ROS generation provoked by Ang II contributes to endothelial dysfunction and oxidation of LDL. Oxidised LDL (oxLDL) favours inflammation by activation of vascular cells and acting as a chemoattractant for monocytes and T-lymphocytes. In fact, its accumulation in the vessel wall is now considered a main trigger for many events seen in early atherosclerosis (Steinberg, 2002). Ingestion of oxLDL by tissue macrophages gives rise to foam cells, a major constituent of atherosclerotic plaques. Additionally, Ang II enhances leukocyte infiltration of the vessel wall by increasing expression of cell adhesion molecules on endothelial cells and VSMCs (Schmidt-Ott *et al.*, 2000).

In endothelial cells, Ang II is capable of triggering apoptosis, and this may contribute to damage in the endothelial cell monolayer (Schmidt-Ott *et al.*, 2000).

4.5.2 Ang II and VSMCs

Molecular effects

Ang II actions in VSMCs extend far beyond vasoconstriction. Through the AT_1-R, Ang II activates a multitude of intracellular pathways that finally lead to altered VSMC function as described below. These pathways include phosphorylation and activation of various kinases and phospholipases (Touyz and Schiffrin, 2000). Pathways and signalling molecules important for this work are discussed separately in discrete chapters.

Long-term effects on VSMC function

Ang II stimulates protein synthesis and causes cellular hypertrophy in cultured VSMCs (Geisterfer *et al.*, 1988; Berk *et al.*, 1989). In the presence of other growth factors, however, Ang II is also capable of inducing hyperplasia in cultured VSMCs, and long term infusion of Ang II in normal and carotid balloon injured rats augmented VSMC proliferation (Daemen *et al.*, 1991). Additionally, Ang II has been shown to activate VSMC migration in transwell-culture chambers (Xi *et al.*, 1999).

In addition to these direct responses, Ang II induces production of various growth factors, cyto- and chemokines, including macrophage chemoattractant protein 1 (MCP-1), interleukin 6 (IL 6), transforming growth factor ß (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), and members of the epidermal growth factor (EGF) family. Except for TGF-β that stimulates collagen synthesis (Libby, 2002), these factors enhance VSMC proliferation and migration and support inflammation (Schmidt-Ott *et al.*, 2000).

IL 6 in particular has been implicated in activation of matrix metalloproteinases (Schmidt-Ott *et al.*, 2000), although direct effects of Ang II on matrix metalloproteinase expression in VSMCs have been reported as well (Takagishi *et al.*, 1995). This is an important aspect of Ang II signalling as metalloproteinase activity in the fibrous cap of atherosclerotic lesion has been associated with plaque rupture (Schmidt-Ott *et al.*, 2000).

Moreover, Ang II triggers changes in extracellular matrix (ECM) composition. Quiescent, contractile VSMCs synthesise little amounts of ECM, but phenotype modulation occurring during vascular remodelling substantially increases synthesis of fibronectin, collagen and elastin. Of interest, these changes in the extracellular environment favour proliferation and migration of VSMCs (Schmidt-Ott *et al.*, 2000).

5 EGF-Receptor transactivation

Besides being activated by ligand binding, the EGF-R can be transactivated by a growing number of different pathways, including GPCRs, cytokine receptors and ion channels (Hackel *et al.*, 1999). In this regard, EGF-R transactivation through the AT_1-R has been proposed to play an important role in early Ang II-signalling (Ushio-Fukai *et al.*, 2001a; Saito *et al.*, 2002; Eguchi *et al.*, 1998).

5.1 EGF-Receptors

The EGF-Receptor (EGF-R, also termed HER1/ErbB-1) is a member of the EGF-R (ErbB/HER) subfamily of receptor tyrosine kinases and was the first receptor tyrosine kinase to be discovered (Carpenter *et al.*, 1978).

It is a 1186 amino acid transmembrane glycoprotein, whose intracellular part can be grouped into three domains: the iuxtamembrane domain serves as a site for feedback attenuation by protein kinase C and Erk 1/2. It is flanked by a kinase domain that is, in turn, followed by a carboxy-terminal tail that functions as an autoinhibitory substrate. Its autophosphorylation is a prerequisite for kinase activation. These phospho-sites are also important for further signal transduction, as they serve as docking sites for signalling molecules containing phospho-tyrosine binding domains (Wells, 1999).

Ligand binding to the extracellular domain induces conformational changes, resulting in the exposure of a receptor-receptor interaction site and, consequently, dimerisation of two ligand-occupied EGF-Rs. Dimerisation is possible with all three other EGF-R family members and facilitates inter-molecular autophosphorylation that finally leads to complete tyrosine kinase activation (Schlessinger, 2002).

5.2 EGF-R transactivation by the AT_1-R

Although the EGF-R is not well characterised in terms of its role in vascular biology, its transactivation has been shown to occur in response to stimulation of the AT_1 receptor in VSMCs (see Fig. 3). Importantly, a role for this link has been established regarding Ang II-induced activation of the mitogen activated protein kinases (MAPKs) extracellular-signal regulated kinase 1/2 (Erk 1/2) and p38 (Eguchi *et al.*, 2001; Frank *et al.*, 2001), and the serine/threonine kinases Akt and p70 S6 kinase ($p70^{S6k}$) (Eguchi *et al.*, 1999b).

Based on the rapid onset of EGF-R phosphorylation as well as the apparent lack of EGF-R ligands in conditioned medium, EGF-R transactivation was initially thought to be mediated exclusively by intracellular events (Daub *et al.*, 1997). Regarding Ang II signalling in VSMCs, the tyrosine kinase c-Src, Ca^{2+}-dependent activation of proline-rich tyrosine kinase 2 (PYK 2) and ROS

have been implicated (Eguchi *et al.*, 1999a; Bokemeyer *et al.*, 2000; Ushio-Fukai *et al.*, 2001a).

More recent studies, however, provide evidence that cleavage of heparin-binding EGF-like growth factor (HB-EGF) from its membrane-anchored precursor is involved in mediating GPCR-induced EGF-R transactivation as well (Kalmes *et al.*, 2001). HB-EGF holds, in addition to its EGF-like unit, a heparin-binding domain that binds to heparan sulfate side chains in cell surface heparan sulfate proteoglycans which serve as co-activators for efficient EGF-R activation. Most interestingly, HB-EGF has been implicated in VSMC hyperplasia and atherosclerosis (Raab and Klagsbrun, 1997).

Additionally, Ushio-Fukai *et al*. have shown an involvement of caveolae and focal adhesions in Ang II-induced EGF-R transactivation (Ushio-Fukai *et al.*, 2001b).

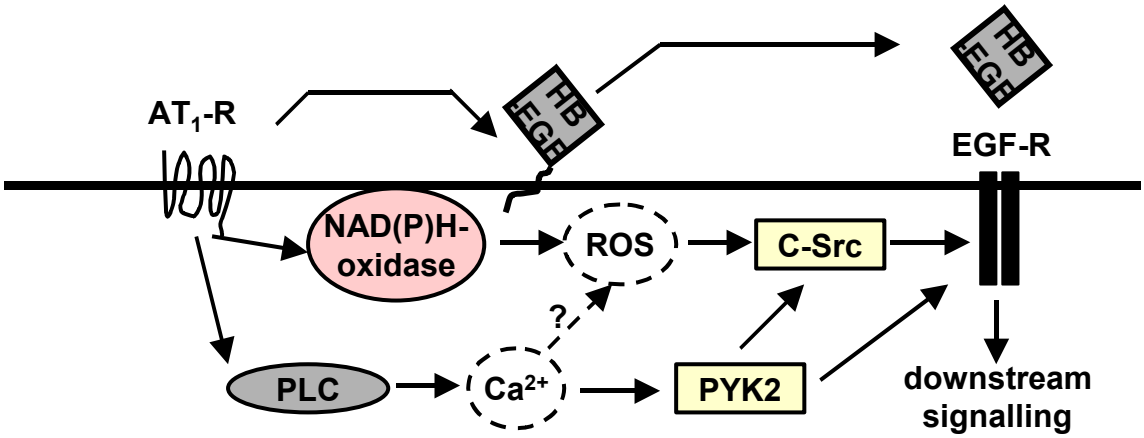

Fig. 3. Mechanisms discussed for Ang II-induced EGF-R transactivation. Intra- as well as extracellular events are required for transactivation. Information for this cartoon was obtained from (Ushio-Fukai et al., 2001a), (Eguchi et al., 2001), and (Eguchi et al., 1999a).

6 Phosphoinositide 3-kinases

6.1 Phosphoinositides

PtdIns consists of a glycerol backbone with fatty acids attached at position 1 and 2 (1-stearoyl, 2-arachidonyl in the naturally occurring form), and an inositol 1-phosphate group at position 3 (Alessi, 2001). In cells, the inositol residue can be phosphorylated in position 3, 4 and 5, and the phosphorylated derivates are then referred to as phosphoinositides (Vanhaesebroeck and Alessi, 2000). Possible cellular functions of these phospholipids are 1), to serve as a substrate for phospholipases to generate soluble IP_3 as second messenger, 2), the

interaction with intracellular proteins and 3) the alteration of membrane topology (Fruman *et al.*, 1998).

6.2 Phosphoinositide 3-kinases- classification and activation

Enzymes capable of transferring phosphates to phosphatidylinositol or phosphoinositides can be divided into three general families: phosphoinositide 3-kinases (PI3-k), phosphatidylinositol 4-kinases and phosphatidylinositol-phosphat kinases. Among the three PI3-k isoforms identified so far, only class I PI3-k have been shown to activate the serine-threonine kinase Akt (see 7.1.) and are therefore relevant for this work (Vanhaesebroeck and Alessi, 2000).

Interestingly, quiescent cells contain significant levels of PtdIns(3)P, but hardly any other 3-phosphorylated phosphoinositides. The latter, however, increase dramatically after cellular stimulation (Vanhaesebroeck *et al.*, 2001). *In vivo*, class I PI3-k seem to phosphorylate predominantly PtdIns(4,5)P_2, generating PtdIns(3,4,5)P_3. The second product of class I PI3-k activity, PtdIns(3,4)P_2, originates most likely from subsequent dephosphorylation accomplished by the phosphatases SHIP 1 and SHIP 2 (Cantley, 2002). Of note, class I PI3-k have also intrinsic protein kinase activity (Fruman *et al.*, 1998).

Class I PI3-kinases can be divided into 2 classes, class IA and class IB. Class IB PI3-k are activated by the $G_{\beta\gamma}$ subunit of heterotrimeric G proteins and show restricted tissue distribution, being abundant only in leukocytes. Class IA PI3-k, in contrast, are widely expressed. They are heterodimeric proteins consisting of a 110 kDa regulatory subunit expressed in three isoforms (p110α, p110β, p110δ) and a 50-85 kDa regulatory subunit of which p85 is the prototype. The regulatory subunit does not have intrinsic kinase activity but provides various sites of interaction with other signalling molecules important for PI3-k activation. It contains a Src homology 3 (SH3) domain, a breakpoint-cluster-region, two proline-rich regions as well as two C-terminal SH2 domains spaced by an inter-SH2 region. The latter mediates tight binding of the p85 subunit to the catalytic subunit (Wymann and Pirola, 1998). Binding of the SH2 domains to phospho-tyrosine, created for example on receptor tyrosine kinases upon growth factor stimulation, targets PI3-kinase to the membrane and increases its activity, suggesting that phosphopeptide-SH2 binding induces conformational strains that propagate to the catalytic subunit *via* the inter-SH2 domain (Wymann and Pirola, 1998).

Apart from recruitment to the plasma membrane and activation *via* its SH2 domains, p85 contributes to activation of class IA PI3-k by binding of the p85 subunit to various other signalling molecules. (Wymann and Pirola, 1998). Whether tyrosine phosphorylation of p85 also contributes to PI3-k activation is discussed controversially (Saward and Zahradka, 1997; von Willebrand *et al.*,

1998). Furthermore, binding of GTP-loaded Ras to the catalytic subunit seems to increase PI3-k activity (Fruman *et al.*, 1998; Wymann and Pirola, 1998).

PI3-k signalling is terminated by the action of PTEN, a phosphatase that dephosphorylates PtdIns(3,4,5)P_3 at position 3. Loss of PTEN has been found in a large fraction of advanced human cancers, indicating that uncontrolled PI3-k signalling contributes to cancer (Cantley, 2002).

6.3 Inhibitors of phosphoinositide 3-kinases

Wortmannin and LY294002 are two structurally unrelated, cell permeable inhibitors of PI3-k. Wortmannin irreversibly inhibits the enzyme by covalent modification of the catalytic subunit (Wymann *et al.*, 1996). LY294002 is a competitive inhibitor of the ATP binding site. Both inhibitors affect other related kinases when applied at concentrations higher than those required for inhibiton of PI3-k (Fruman *et al.*, 1998).

7 Akt, p70^{S6} kinase and MAPK

7.1 Akt kinase

Akt (also termed PKB due to sequence homologies with PKA and PKC) is the cellular homologue of the transforming oncogene of the AKT8 oncovirus and exists in 3 isoforms (Datta *et al.*, 1999; Scheid and Woodgett, 2001). It is a serine/threonine kinase whose activation depends on the generation of 3-phosphorylated phosphoinositides generated by class I PI3-kinases. These lipids interact with the pleckstrin homology (PH) domain present in the amino-terminus of Akt, thereby recruiting Akt to the membrane (Scheid and Woodgett, 2001).

Translocation brings the enzyme into proximity with other PH-domain-containing enzymes such as the protein kinase PDK 1 (3-phosphoinositide-dependent kinase 1). This kinase phosphorylates Akt at threonine^{308} in the activation loop, thereby enabling binding of ATP and substrate. For full kinase activation, additional phosphorylation on serine^{473} is necessary, but the kinase responsible, termed PDK 2, is still not identified. Possible candidates are PDK 1 and Akt itself (Toker and Newton, 2000; Brazil and Hemmings, 2001) (see Fig. 4).

Akt kinase is inactivated by the action of the serine/threonine phosphatase PP2A (Millward *et al.*, 1999).

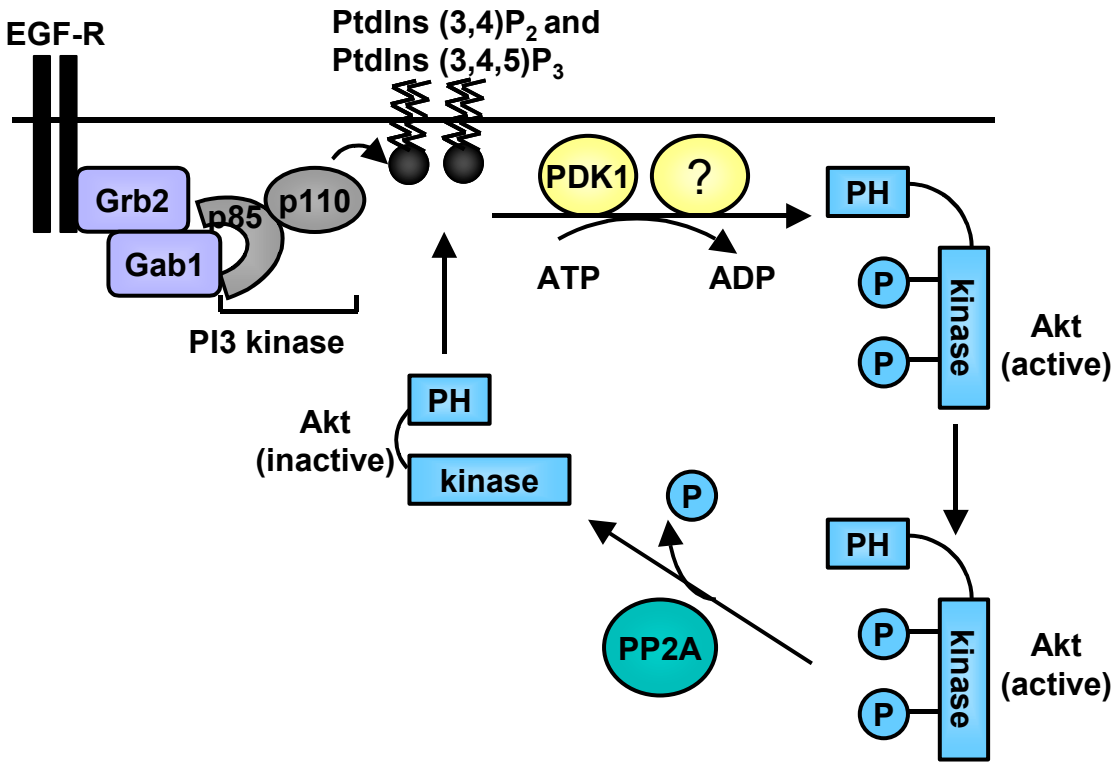

Fig. 4. Aktivation of Akt kinase downstream of the EGF-R. Recruitment of PI3-k to the activated EGF-R via the adapter complex Grb 2/Gab 1 generates 3-phosphorylated phosphoinositides on the membrane. These recruit Akt via its PH domain. At the membrane, Akt is phosphorylated by PDK 1 and a yet unidentified kinase to yield active Akt that translocates to the cytosol and the nucleus. Adapted from (Vanhaesebroeck and Alessi, 2000).

One of the first Akt substrates (for overview, see Fig. 5) identified was glycogen synthase kinase 3 (GSK 3). Phosphorylation inactivates GSK 3, and therefore stabilises or activates GSK 3 targets such as cyclin D, c-Myc and glycogen synthase (Cohen and Frame, 2001).

Akt favours cell survival by phosphorylation of the pro-apoptotic protein BAD and caspase 9 (Datta *et al.*, 1999).

Additional Akt targets comprise the FOXO family of forkhead transcription factors. Phosphorylation of these transcription factors that enhance for example transcription of the cell cycle inhibitor $p27^{Kip1}$, results in inactivation by cytoplasmatic retention or inhibition of DNA binding (Burgering and Kops, 2002; Scheid and Woodgett, 2001).

Akt promotes protein synthesis by phosphorylation and activation of mTor (mammalian target of rapamycin) and GSK 3-dependent activation of eukaryotic initiation factor 2B (eIF2B) (Scott *et al.*, 1998; Cohen and Frame, 2001).

Akt-mediated phosphorylation and inactivation of Raf is the basis for cross-talk between the Akt and the Erk 1/2 pathway and leads to inhibition of the latter (Zimmermann and Moelling, 1999; Reusch *et al.*, 2001).

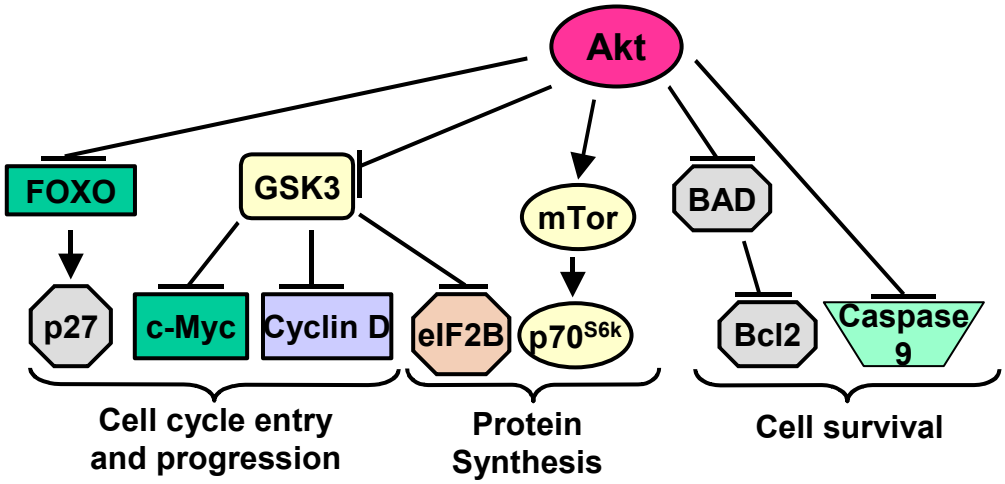

Fig. 5. Selected Akt targets and their impact on cell physiology. Akt activity results in either activation (->) or inhibition (-|) of its immediate downstream targets.

7.2 p70^{S6k}

p70^{S6K} and its nuclear 85 kDa isoform are serine/threonine kinases that phosphorylate the S6 protein of the small 40S subunit of the ribosome at multiple sites. Since the discovery of a new homologue, S6K2, both isoforms are now termed S6K1 (Dufner and Thomas, 1999).

Phosphorylation of the S6 protein increases translation of mRNAs that have an oligopyrimidine tract at their transcriptional start site (Dufner and Thomas, 1999), although recent data suggest translational activation of these mRNAs independent of S6K1 activity and S6 phosphorylation (Stolovich *et al.*, 2002). mRNAs containing an oligopyrimidine tract encode for many components of the translational apparatus (Pullen and Thomas, 1997).

p70^{S6k} activation requires multiple phosphorylation accomplished by various kinases, including mTOR, atypical PKC and PDK 1 (Dufner and Thomas, 1999; Scott *et al.*, 1998).

In VSMCs, rapamycin-inhibitable Ang II mediated activation of p70^{S6k} has been shown to be implicated in protein synthesis (Giasson and Meloche, 1995).

7.3 MAPK

MAPK are ubiquitously expressed serine/threonine kinases that control a variety of physiological processes, including cell growth, transformation, differentiation and apoptosis. For activation, these kinases require dual phosphorylation on threonine and tyrosine. Inactivation occurs by the action of specific MAPK phosphatases. In this way, the balance between phosphorylation and dephosphorylation regulates MAPK activity, allowing the cell to rapidly adjust to environmental changes (Johnson and Lapadat, 2002).

MAPK are part of a signalling cascade that consists of three subsequently activated kinases: MAPK are phosphorylated and activated by MAPK kinases (MKK or MEK), which are in turn activated by MAPK kinase kinases (MKKK or MEKK) (see Fig. 6). Main targets of the MAPK are other protein kinases, phospholipases and, of importance, transcription factors (Johnson and Lapadat, 2002).

So far, there are three well characterised subfamilies of MAPK:

1) the extracellular-signal regulated kinases Erk 1 and Erk 2. Erk 1/2 is essentially involved in proliferation after stimulation with mitogenic agents. Targets include the transcription factors Elk 1 and c-Myc and the p90 ribosomal S6 kinase (Takahashi and Berk, 1998).

2) the c-Jun N-terminal kinases (JNK) 1, 2 and 3. JNKs are stress-activated protein kinases that activate the transcription factor c-Jun, a component of the AP-1 transcription factor (Takahashi and Berk, 1998).

3) four p38 isoenzymes termed p38α, β, γ and δ. p38 is activated by a similar range of stimuli than JNK, and seems to overlap with JNK in some but not all functions (Takahashi and Berk, 1998).

In VSMCs, Ang II leads to a rapid activation of all three MAPK subfamilies. An additional MAPK familiy, Erk 5 (BMK1) has been recently identified (Takahashi and Berk, 1998).

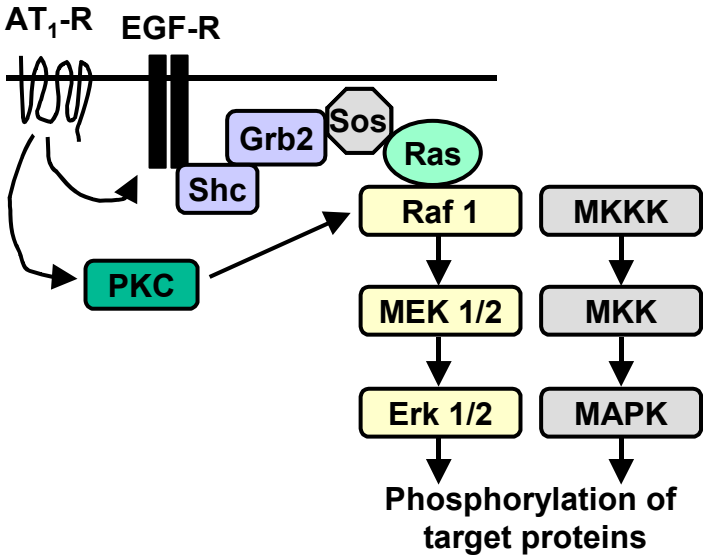

Fig. 6. The MAPK cascade, explained by the example of Ang II mediated activation of the MAPK Erk 1/2 in VSMCs. Binding of Ang II to the AT_1-R leads to transactivation of the EGF-R (see 5.2) that recruits adapter proteins such as Shc and Grb 2. Grb 2 is constitutively associated with the Guanin-nucleotide exchange factor Sos that activates the small molecular weight GTP-binding protein Ras. Ras, in its GTP-bound state, recruits and activates the MKKK Raf-1, initiating the MAPK cascade. Additionally, Ras-independent, PKC-dependent pathways of Erk 1/2 activation have been described (Takahashi et al., 1997; Liao et al., 1997).

8 c-Src kinase

c-Src is the cellular counterpart of the retroviral oncogene v-Src, discovered more than 30 years ago in the Rous sarcoma virus (Martin, 2001). It is a part of the Src family of non-receptor tyrosine kinases that consists of 9 members, although only c-Src, Fyn and Yes are ubiquitously expressed. Myristoylation at the N-terminus mediates membrane localisation. Other functional domains are, starting from the N-terminus, a unique domain, a SH3 domain, a SH2 domain, a catalytic domain and a short C-terminus that is truncated in v-Src and essential for kinase regulation (Hubbard and Till, 2000; Martin, 2001).

c-Src kinase activity is regulated in diverse modes (seen Fig. 7). Phosphorylation at tyrosine^{529} (numbers are specific to human c-Src) in the C-terminus results in kinase inactivation caused by intramolecular interactions with the SH2 domain. This inhibitory interaction can be abolished by dephosphorylation, by phosphorylation of tyrosine^{215} in the SH2 domain or by binding of the SH2 domain to a ligand with greater affinity (Martin, 2001; Stover *et al.*, 1996). Interaction of the SH3 domain and the linker between the SH2 domain and the kinase domain further stabilise the inactive conformation. Similar to the SH2 domain, displacement due to binding of a ligand with higher affinity to SH3 contributes to kinase activation (Abram and Courtneidge, 2000).

Disrupting the inactive conformation by perturbation of intramolecular interactions permits intermolecular autophosphorylation of tyrosine[418], an event that is necessary for full kinase activation (Abram and Courtneidge, 2000).

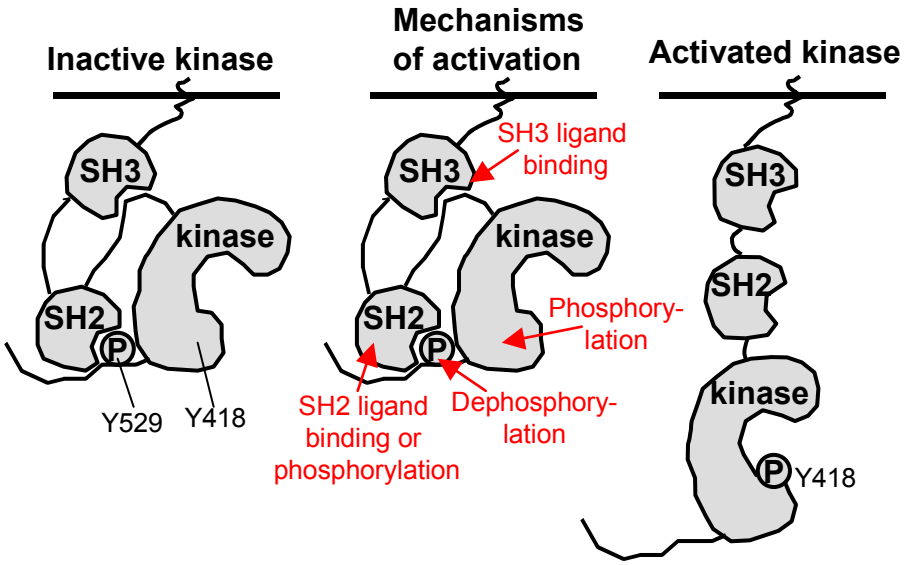

Fig. 7. Regulation of c-Src kinase activity. Adapted from (Martin, 2001).

9 Cell Cycle

The eucaryotic cell cycle consists of four distinct stages: G1-phase (presynthetic), S-phase (synthetic), G2-phase (premitotic) and M-phase (mitotic) (see Fig. 8). Quiescent cells are in so-called G0-phase and enter the cell cycle upon mitogenic stimulation. In the G1-phase of the cell cycle, mitogenic and anti-mitogenic signals are integrated and the decision is made whether to continue, exit or pause the cell cycle. In this regard, hyperphosphorylation of the Rb by cyclin E-dependent kinases is an important checkpoint. Once overcome, the cells are committed to further progress through the cell cycle, independent of extracellular stimuli (Johnson and Walker, 1999).

9.1 Regulation of G1-phase

G1-phase is under the control of so-called cyclin-dependent kinases (cdk). These assemble with cyclins, proteins that are sequentially synthesised and degraded throughout the cell cycle. To be fully activated, cdk/cyclin complexes require phosphorylation by a cdk-activating kinase (CAK) after entering the nucleus (Sherr and Roberts, 1999).

D-type cyclins are the first to be induced after exposure of cells to mitogenic stimuli: Erk 1/2 increases transcription of the cyclin D gene and post-translationally regulates its assembly with cdk4 or 6. Activated Akt, on the other

hand, prevents cyclin D degradation by inhibiting its GSK 3-dependent phosphorylation and subsequent degradation. Thus, mitogens exert their impact on cell cycle progression predominantly *via* D-type cyclins (see also Fig. 8).

The cyclin D-dependent kinases cdk4 and 6 phosphorylate the Rb, that is part of the pocket protein family (Sherr and Roberts, 1999). Rb, in its hypophosphorylated, active state, binds to promoter-bound members of the E2F family of transcription factors, inhibiting their transcriptional activation and, thus, transcription of E2F-responsive genes. These encode for many components of the replication machinery, including cyclin E and DNA polymerase α. Additionally, Rb actively represses transcription by recruiting histone-modifying enzymes such as histone deacetylases and a histon methylase (Nielsen *et al.*, 2001; Trimarchi and Lees, 2002; DePinho, 1998). Phosphorylation of Rb, which releases E2F from transcriptional restraint, is initiated by cyclin D/cdk4/6 and completed by cyclin E/cdk2 (Sherr, 1996).

Cyclin E/cdk2 complexes have broader substrate specificity and phosphorylate, in addition to Rb, also several proteins at replication origins as well as the cdk-inhibitor $p27^{Kip1}$, leading to degradation of the latter (Sherr, 1996). The second cyclin partner for cdk2, cyclin A, is synthesised not before late G1, and although cyclin A/cdk2 complexes share some targets with cyclin E/cdk2, they additionally inhibit E2F-binding to DNA in S-phase (Sherr, 1996).

9.2 Inhibitors of cyclin-dependent kinases

There are two families of cyclin-dependent kinase inhibitors. INK4 proteins (Inhibitors of CDK4) specifically inhibit the catalytic subunits of cdk4/6. In contrast, the so-called Cip/Kip proteins, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, affect the catalytic activity of cyclin D-, E- and A-dependent kinases by binding of both the catalytic and the cyclin subunit (Sherr and Roberts, 1999).

For a long time, Cip/Kip proteins were thought to be exclusively inhibitory, but recent data provide a more differentiated view: although still being considered potent inhibitors of cyclin A- and E-dependent cdk2, there is increasing evidence that Cip/Kip proteins act as positive regulators of cyclin D-dependent kinase activity (Cheng *et al.*, 1999). Interestingly, this hypothesis implies that cyclin D-dependent kinases contribute to cell cycle progression not only by phosphorylating Rb but also by complexing Cip/Kip proteins, thereby leading to an indirect activation of cyclin E- and A-dependent kinases (Cheng *et al.*, 1999).

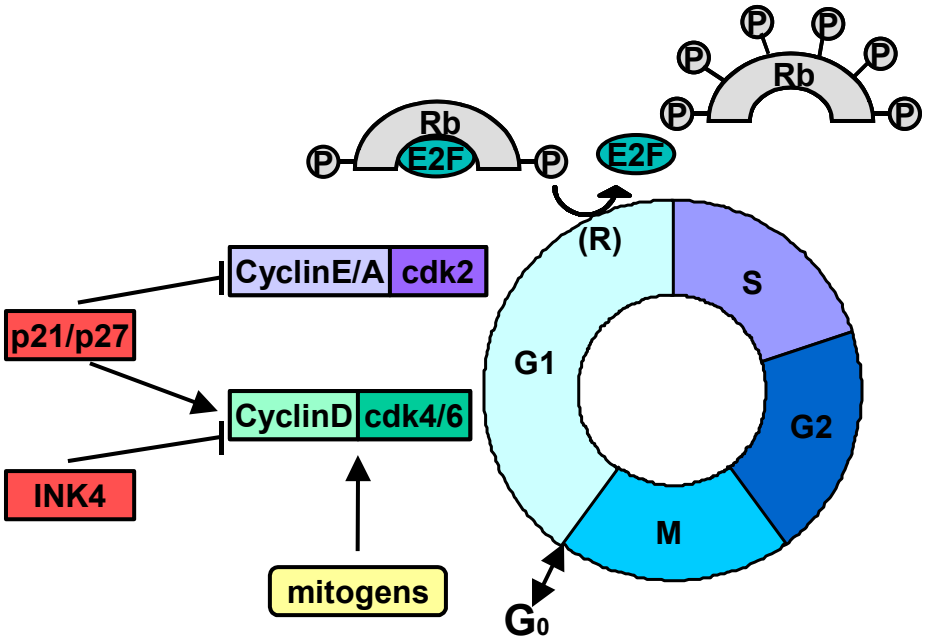

Fig. 8. Cell cycle phases and proteins involved in regulation of G1-phase. R = restriction point

9.3 p53

p53 is a prototype checkpoint protein, not required for normal cellular proliferation but indispensable for the ability to arrest cell cycle progression in response to stress conditions. In fact, it is the gene most frequently altered in human cancers, with an incidence of about 50% (Vousden and Lu, 2002).

Regulation of p53 protein levels occurs predominantly by post-translational mechanisms. The major player in regulating p53 protein levels is Mdm2. This protein inhibits transcription of p53-regulated genes by binding to the transactivation domain of p53. Moreover, it functions as a p53-specific E3 ubiquitin ligase, promoting p53 degradation and limiting its half life to sometimes as short as a few minutes. Interestingly, Mdm2 is part of an autoregulatory loop as it is itsself a p53-regulated gene (Oren, 1999). Disruption of the Mdm2/p53 interaction, for example by phosphorylation of p53 in its Mdm2 binding region (Schon *et al.*, 2002) or by binding of $p19^{ARF}$ to Mdm2, stabilises p53 (Sherr, 2001; Sherr, 1998; Oren, 1999).

p53–regulated genes are numerous and involved in apotosis, cell-cycle arrest and DNA repair, angiogenesis and autoregulation. In apoptosis, contribution of p53-mediated transcriptional repression is discussed as well (Vousden and Lu, 2002).

Regulation of the final outcome of p53 activation in a cell, namely apoptosis or just cell cycle arrest, is very complex and only partially understood: different

p53-affinity of the various promoters, covalent modification of the p53 protein, subcellular localisation as well as the availability of cofactors and p53-binding proteins that may contribute to the apoptotic response seem to play a role (Ryan and Vousden, 2002; Vousden and Lu, 2002).

9.4 The cell cycle as a therapeutic target in cardiovascular disease

Mitogenic stimuli trigger different signalling pathways, but all finally converge at the cell cycle. Therefore, the cell cycle provides a promising target for therapeutic intervention, reflected also by the various approaches that have been made in recent years, especially for preventing in-stent restenosis or bypass graft failure. One strategy was to limit VSMC proliferation by application of antiproliferative drugs such as heparins, ACE-inhibitors or growth factor antagonists, but clinical trials generally failed to reproduce results obtained in animal studies (Dzau *et al.*, 2002).

Local delivery of cytostatic or cytotoxic agents has proved more successful. Although radiotherapy and paclitaxel are able to reduce stenosis (Grise *et al.*, 2002; Teirstein *et al.*, 1997; Dzau *et al.*, 2002), so far, the most promising agent seems to be rapamycin (Sirolimus). This substance selectively inhibits mTor and exerts its antiproliferative properties by reduction of translation and stabilisation of $p27^{Kip1}$(Vinals *et al.*, 1999; Graves *et al.*, 1995; Dzau *et al.*, 2002). In a recent trial comparing rapamycin-coated stents to uncoated stents, no restenosis (as defined by luminal narrowing of at least 50%) was seen in the rapamycin group, in contrast to 27% in the control group after 6 months (Morice *et al.*, 2002).

A third strategy is local gene therapy, and antisense oligodeoxynucleotides (ODN) for cell cycle regulatory genes such as c-myc, PCNA and different cdks have been successfully tried in animal models (Dzau *et al.*, 2002). Moreover, decoy ODNs for E2F have proved save and efficacious in human trials (Mann *et al.*, 1999; Mangi and Dzau, 2001; Dzau *et al.*, 2002).

Taken together, local application of agents capable of preventing VSMC proliferation seems to be a promising approach for preventing restenosis and vein bypass graft failure. However, this strategy is probably not feasible for treatment of atherosclerosis in general, as proliferation, in that case, is not temporally and spatially limited. Moreover, there is considerable risk that apoptosis of plaque VSMCs may result in plaque instability and rupture (Dzau *et al.*, 2002).

C. Materials and Methods

1 Preparation of major stock solutions

Preparation of Ang II solution

Ang II (Sigma) was dissolved in 0.25% BSA solution (Roche) and stored at -80°C as a 2 mM stock solution. Working aliquots were obtained by diluting stock solutions in 0.25% BSA to a concentration of 20 µM and stored at –20°C. For experiments, 100 nM Ang II were used.

Preparation of EGF solution

100 µg/ml stock solutions of EGF (Upstate) were prepared in PBS and stored at –20°C. For experiments, 100 ng/ml EGF were used.

Calf serum

Calf serum (Life Technologies) was heat inactivated for 40 minutes at 55°C and stored at –20°C.

Resveratrol

50 or 100 mM stock solutions of *trans*-Resveratrol (Sigma) were prepared in DMSO and stored in 10 µl aliquots at –20°C.

2 Cell culture

2.1 Solutions

PBS:

Na_2HPO_4		1.48 g
KH_2PO_4		0.43 g
NaCl		7.20 g
H_2O	ad	1000 ml

PBS+

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_20		0.10 g
$CaCl_2$		0.10 g
H_2O	ad	1000 ml

Trypsin/EDTA:

Trypsin (diluted 1:250 in PBS)		0.05 g
EDTA		0.20 g
PBS	ad	100 ml

Digestion buffer

Collagenase (253 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 ml

2.2 Cell isolation

VSMCs were isolated from male Sprague-Dawley rat (Charles River Wiga) thoracic aortas by enzymatic digestion. Aortas were removed and placed in PBS^+ for further processing. Briefly, they were cut open and placed in digestion buffer (adapted from (Palmberg *et al.*, 1985) for 15 minutes at 37°C before scraping off the endothelium and adventitia. Aortas were then minced and incubated in digestion buffer for an additional 4 hours at 37°C. After centrifugation, cells were placed in a 25 cm^2 flask and grown in DMEM without phenol red (Bio Whittaker) supplemented with 10% calf serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (growth medium). A minimum of four aortas is needed for one 25 cm^2 flask. VSMCs were identified by FACS analysis and 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 (HUVEC), Raw 264.7 cells and L929 murine fibroblasts were used as negative controls to test the specifity 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.

2.3 Passaging of VSMCs

Cells were passaged twice a week. Growth medium was removed and cells were washed once with 2 ml of Trypsin/EDTA solution before incubation with Trypsin/EDTA solution for approximately 5 minutes to allow detachment of the cells. Cells were subcultured 1:5 in 75 cm^2 culture flasks or in appropriate plates. For experiments, cells between passage 7 and 15 were cultured in 6- or 24-well plates or 60 mm dishes and used at 70-90% confluence.

When Ang II or EGF were used for stimulation, cells were serum-starved in Dulbecco`s Modified Eagle`s Medium (DMEM) containing 0.1% calf serum over night prior to experiments. For serum-stimulation, cells were made quiescent by incubation in DMEM without calf serum for at least 24 hours.

2.4 Freezing, storage and thawing of VSMCs

For freezing, VSMCs from one 75 cm^2 flask were trypsinised, spun down and resuspended in ice-cold freezing medium (1.1 ml DMSO, 8 ml DMEM, 2 ml calf serum). 1 ml aliquots were frozen in cryovials first at -20°C for one day, then at -80°C over night 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 cm^2 flask.

3 Detection of cell death and cell viability

When substances such as resveratrol are used for experiments, induction of cell death should be ruled out as a possible cause of their molecular effects. Accordingly, we performed various experiments to make sure that resveratrol showed no cytotoxicity at the concentrations used. Since resveratrol displayed no cytotoxicity in any of the tests listed below, the data are not shown in the Results section.

Two distinct forms of eukaryotic cell death can be distinguished by morphological and biochemical criteria: necrosis and apoptosis.

Necrosis is characterised by cell swelling and plasma membrane rupture, denaturation and coagulation of cytoplasmatic proteins and breakdown of cell organelles. It is caused, for example, by stresses such as ischemia or chemical injury.

Apoptosis is, in contrast to necrosis, a tightly controlled process that is triggered by activation of a cellular suicide programme. It is accompanied by membrane blebbing, condensation of cytoplasm and the activation of a Ca^{2+}- and Mg^{2+} dependent endonuclease as well as specific proteases.

3.1 Apoptosis

3.1.1 Staining of apoptotic nuclei with Hoechst 33342

The bisbenzimidazole Hoechst 33342 is a cell permeable adenine-thymine specific fluorescent dye. Staining of apoptotic nuclei with Hoechst 33342 leads to bright blue staining of condensed chromatin that can be detected by fluorescence microscopy (Dive *et al.*, 1992).

Cells grown in 6-well plates were serum-starved over night and treated with 50 µM (for Ang II) or 100 µM resveratrol (for calf serum) for 30 minutes prior to addition of Ang II or 10% calf serum. After 24 hours, medium was replaced by 1 µg/ml Hoechst 33342 in PBS and cells were incubated at 37°C for 10 minutes. Subsequently, the dye was removed by washing with PBS and cells were visualised by fluorescence microscopy using 365 nm as excitation wavelength (Axiovert 25, Carl Zeiss, Jena, Germany).

3.1.2 Nucleosome ELISA

Fragmentation of the genomic DNA is a hallmark of apoptosis. The above-mentioned endonuclease cleaves double-stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. Nucleosomal DNA is tightly complexed with core histones and is therefore protected from cleavage by the endonuclease.

Cell death detection ELISA^{PLUS} (Roche) was used to detect mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. The assay is based on a quantitative sandwich–enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. The experiment was carried out as described in the manufacturer's manual. Briefly, cells were seeded at low density in 96-well plates and grown for 48 hours, serum-starved over night and subsequently stimulated with or without 10% calf serum in the presence or absence of various concentrations of resveratrol. 24 hours later cells were lysed and the lysate centrifuged for 10 minutes at 200 g to remove cell nuclei containing unfragmented DNA. An aliquot of the supernatant was transferred to a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin antibody and anti-DNA-peroxidase antibody. A positive control provided by the company was included. After two hours of gentle shaking, unbound antibodies were carefully removed by a multiple washing step before addition of the peroxidase substrate 2,2`azino-di-[3-ethylbenzthiazoline sulphonate (6)] (ABTS) and incubation for 20 minutes. Absorption was measured in an SLT spectra ELISA reader (SLT lab instruments, Crailsheim, Germany) at 415 nm against ABTS solution as blanc.

3.2 Necrosis

Lactate dehydrogenase (LDH) activity

LDH activity was assessed in the medium of cells treated with resveratrol. As mentioned before, necrotic cell death leads to plasma membrane rupture. Cytoplasmatic enzymes such as LDH can therefore be detected in the supernatant. LDH catalyses the conversion of pyruvate to lactate. Concomitantly, NADH is oxidised to NAD^+, and the resultant decrease of NADH can be assessed photometrically (Bergmeyer H.U., 1974).

Phosphate buffer

K_2HPO_4 (8.74 g/l) and KH_2PO_4 (6.8 g/l) solutions were used to obtain a solution with a pH of 7.5. Subsequently, 66 mg of pyruvate were added to 1 litre of buffer.

NADH solution

NADH-Na_2	10 mg
$NaHCO_3$ (0.5%)	1 ml

Cells were grown in 60 mm dishes, kept in DMEM supplemented with 0.1% calf serum over night and treated with resveratrol in different concentrations or vehicle for 30 minutes prior to stimulation with or without Ang II for 24 hours. 600 µl of supernatant were mixed with 400 µl of phosphate buffer before addition of 10 µl of NADH solution. Enzyme activity was calculated based on the

decrease in NADH extinction (ε_{365nm} = 3.34 mM^{-1} cm^{-1}) measured with a Lambda Bio 20 photometer (PerkinElmer).

3.3 Cytotoxicity/ Cell viability

3.3.1 Propidium iodide exclusion assay

Differential staining with propidium iodide (PI) can be used to investigate plasma membrane integrity of cells. In cells with intact plasma membrane, the DNA-intercalating dye PI is not able to enter the cell without prior permeabilisation. In damaged cells, however, PI pervades the plasma membrane and bright nuclear fluorescence can be detected. Accordingly, in flow cytometry, an increase in FL2 fluorescence can be observed.

PI solution

PI		2.5 µg
PBS	ad	1 ml

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

3.3.2 MTT cell viability assay

In this assay, the metabolic activity of cells is assessed. Mitochondrial reduction of the yellow-coloured 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to the corresponding purple formazan can be taken as an indicator of cell viability (Mosmann, 1983).

MTT solution

MTT		5 mg
PBS	ad	1 ml

Cells were grown in 96-well plates for 48 hours, serum-starved over night and treated with various concentrations of resveratrol or vehicle before addition of Ang II or calf serum for 24 hours. Medium containing resveratrol was then carefully replaced to avoid direct reactions between resveratrol and MTT. MTT solution was added to each well to reach a final concentration of 0.5 mg/ml and incubated for 5 hours at 37°C. Subsequently, 190 µl of DMSO were added and the plate shaken for 2 hours in the dark. Absorption was measured in an SLT spectra ELISA reader (SLT labinstruments) at 550 nm.

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. pH at 4°C with NaOH to pH 7.5. Bring final volume up to 430 ml with H_2O. Store at 4°C

Prepare 1x lysis buffer just before use:

Stock concentrate	8.6 ml
PMSF	1 mM
Complete® 25 x (Roche)	400 µl
Triton X-100 (10% in H_2O)	1 ml

SDS sample buffer (stock solution)

TRIS-HCl (pH 6.8)	37.5 ml
SDS	6 g
Glycerol	30 ml
Bromphenol blue	15 mg

SDS sample buffer (3 x)

Stock solution	850 µ
ß-mercaptoethanol	150 µl

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

Resolving gel 10%		Stacking gel	
PAA solution 30%	5.0 ml	PAA solution 30%	1.7 ml
TRIS-base pH 8.8	5.75 ml	TRIS-HCl, pH 6.8	1 ml
SDS 10%	0.15 ml	SDS 10%	0.1 ml
H_2O	6.1 ml	H_2O	7.0 ml
TEMED	15 µl	TEMED	20 µl
APS	75 µl	APS	100 µl

Gels were prepared using a 30% solution of PAA/0.8% bisacrylamide. Different concentrations of PAA in the resolving gel were used according to the molecular weight of the protein of interest. Before addition of TEMED and APS, the solutions were vacuum-degassed for 10 minutes.

Electrophoresis buffer (5 x)

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

Blotting buffer (5 x)			Blotting buffer (1 x)	
TRIS-base		15.2 g	Blotting buffer (5 x)	200 ml
Glycine		72.9 g	Methanol	200 ml
H_2O	ad	1000.0 ml	H_2O	600 ml

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

TRIS-base		3.0 g
NaCl		11.1
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 ml
Ethanol (96%)		225 ml
H_2O	ad	500 ml

Coomassie destaining solution

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

4.2 Procedure

Sample preparation

Cells grown in 60 mm dishes were stimulated with Ang II, EGF or calf serum for the indicated times. If inhibitors were used (Table 1), these were preincubated for 30 minutes.

When the phosphatase inhibitor okadaic acid was used, cells were pretreated with resveratrol, wortmannin or vehicle for 15 minutes and subsequently treated with okadaic acid for another 30 minutes prior to stimulation.

For γ-irradiation experiments, cells were treated with 10 Gy for 12 hours before lysis.

Cells were harvested on ice by washing twice with ice-cold PBS and addition of 200 µl lysis buffer. After 30 minutes of incubation on ice, cells were scraped off and the lysates were transferred to pre-cooled eppendorf tubes. The homogenates were cleared by centrifugation at 14,000 g for 10 minutes at 4°C, the supernatants aliquoted and mixed with 3 x lysis buffer. Samples were boiled at 95°C for five minutes and stored at -85°C. Protein concentration in the supernatant was determined by the method of Bradford (Bradford, 1976).

Inhibitors used:

Name	Concentration	Provider
AG 1478	250 nM	Qbiogene-Alexis
Aphidicolin	1 µM	Calbiochem
ICI 182,780	1 µM	Tocris
NAC	10 mM	Sigma
Ocadaic acid	1 µM	Acros Organics
PD98,059	20 µM	Qbiogene-Alexis
PP1	20 µM	Qbiogene-Alexis
Wortmannin	50 nM	Qbiogene-Alexis

Table 1: inhibitors

Electrophoresis, tank blotting and protein detection

Equal amounts of protein (60 µg for Retinoblastoma protein, 20 µg for all other proteins) were loaded and separated by SDS-PAGE (Mini PROTEAN 3, Bio-Rad). Proteins were then transferred for 60 minutes at 100 V to a nitrocellulose membrane (Hybond™ ECL™, Amersham Biosciences) with a Mini Trans-Blot® Cell (Bio-Rad). After blocking with 5% non-fat dry milk (Bio-Rad) in TBS-T for one hour, primary antibodies (Table 2) dissolved in TBS-T containing 5% BSA were added over night at 4°C. The next day, membranes were incubated with appropriate horseraddish-peroxidase conjugated secondary antibodies (Table 3) for one hour at room temperature. Proteins were detected using an enhanced chemiluminescence protein detection kit (NEN) and a Kodak Digital Science image station 440 cf (PerkinElmer, Rodgau-Jügesheim, Germany).

Primary antibodies used:

Target	Source	Dilution	Provider
Akt	rabbit	1:2000	Cell Signaling
phospho-Akt (ser^{473})	rabbit	1:2000	Cell Signaling
Bax	mouse	1:1000	BD Biosciences
Erk 1/2	rabbit	1:5000	Cell Signaling
phospho-Erk 1/2	rabbit	1:5000	Cell Signaling
EGF-R	rabbit	1:1000	Cell Signaling
phospho-EGF-R (tyr^{845})	rabbit	1:1000	Cell Signaling
p21	mouse	1:1000	BD Biosciences
p27	mouse	1:1000	BD Biosciences
phospho-p38	rabbit	1:1000	Cell Signaling
p53	mouse	1:1000	Cell Signaling
phospho-p53 (ser^{15})	rabbit	1:1000	Cell Signaling
p70	rabbit	1:2000	Cell Signalling
phospho-p70 (thr^{421}/ser^{424})	rabbit	1:2000	Cell Signalling
p-Tyr-100	mouse	1:2000	Cell Signaling
PI 3-kinase p85	rabbit	1:4000	Upstate
Src	rabbit	1:1000	BioSource
phospho-Src (tyr^{215})	rabbit	1:1000	BioSource
phospho-Src (tyr^{418})	rabbit	1:1000	BioSource
Retinoblastoma protein	mouse	1:1000	BD biosciences

Table 2: primary antibodies

Secondary antibodies used:

Target	Source	Dilution	Provider
rabbit IgG	Goat	1:20000	Jackson
mouse IgG1	Goat	1:1000	Serotec

Table 3: secondary antibodies

Coomassie blue staining

In order to control equal loading and transfer of proteins, gels were stained with Coomassie blue staining solution after blotting. Briefly, gels were incubated in staining solution for 30 minutes and subsequently washed 3 times for 10 minutes in destaining solution.

5 Immunoprecipitation

Lysates were prepared as described in the Western blot section. After centrifugation, protein concentration in the supernatant was determined by the bicinchoninic acid assay method (Smith *et al.*, 1985). Supernatant containing equal amounts of protein (200 µg for immunoprecipitation of PI3-k p85 subunit,

500 µg for EGF-R immunoprecipitation) were transferred to pre-cooled eppendorf tubes and lysis buffer was added to reach a final protein concentration of 1 µg/µl. 2 µl of anti-p85 or 5 µl of anti-EGF-R antibody (see Table 2) were added and samples were mixed overnight at 4°C. Antibodies were precipitated by incubation with 50 µl of washed protein A-agarose beads (Sigma) for 2 hours followed by centrifugation. The beads were washed three times with 500 µl of lysis buffer and resuspended in 25 µl of 3 x sample buffer (see 4.1). After addition of 25 ml of 1 x sample buffer, samples were boiled at 95°C for 5 minutes and beads removed by centrifugation. 30 µl (for phospho-tyrosine) or 10 µl (for p85 or EGF-R) of the samples were separated on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane (100 V, 90 minutes). Detection of proteins was performed as described under 4.2.

6 Assessment of VSMC hypertrophy

Hypertrophy is characterised by an increase in cell size but not in cell number. Therefore, protein synthesis can be taken as an indicator of hypertrophy as long as proliferation is excluded as a cause of increased protein synthesis. Ang II is known to induce hypertrophy but not hyperplasia in cultured VSMCs (Geisterfer et al 1998).

6.1 [³H]leucine incorporation

To measure protein synthesis, VSMCs grown in 6-well plates were made quiescent for 48 hours in DMEM containing 0.1% calf serum. After pre-treatment with vehicle, resveratrol, wortmannin or PD 98,059 at the indicated concentrations for 30 minutes, cells were incubated with [³H]leucine (1 µCi/ml, Amersham) in the presence or absence of 100 nM Ang II for 24 hours and the amount of incorporated [³H]leucine was assessed. Briefly, cells were washed twice with ice-cold PBS and incubated with ice-cold 5% trichloroacetic acid for 5 minutes at 4°C. After two additional washes, cells were dissolved in 1 ml 0.4 M NaOH. Dublicate aliquots (0.4 ml) were transferred to scintilation vials, acidified with 0.2 ml of 1.0 M HCl and counted in 5 ml Liquiscint (Roth) in a LS 6500 Beckman Coulter (Beckman).

6.2 Cell counting

To exclude that increase in [³H]leucine incorporation upon Ang II stimulation was due to increased proliferation, cells were seeded at a density of 50,000 cells/well in 6-well plates, grown for 48 hours and made quiescent by incubation with DMEM containing 0.1% calf serum over night. Cells were then left untreated or stimulated for 24 hours with either 100 nM Ang II or 10% calf serum before counting in a Fuchs-Rosenthal-Chamber.

7 Cell cycle analysis

7.1 PI staining

PI is a DNA intercalating dye. The intensity with which a cell`s nucleus emits fluorescence light is direct proportional to the amount of bound PI and therefore to the cell`s DNA content. In this experiment, cells are permeabilised with Triton X-100, enabling PI to enter the cells. Flow cytometry permits individual evaluation of cells as they pass a laser beam in a single file within a fluid stream.

Hypotonic fluorochrome solution (in PBS):

0.1% (v/v) Triton X-100

0.1% (w/v) sodium citrate

50 µg/ml PI

Cells were seeded at a density of 50,000 cells/well in 6-well plates and kept in growth medium for minimum 24 hours. Before experiments, cells were serum-starved for at least four days to ensure synchronisation in G0-phase of the cell cycle.

For cell cycle analysis, cells were treated with 10% calf serum for different time periods. For studies involving resveratrol, cells were either left untreated or pre-treated with various concentrations of resveratrol for 30 min before stimulation with 10% calf serum. Twenty-two hours later cells were trypsinised, washed once with PBS and resuspended in a hypotonic fluorochrome solution. After incubation at 4°C over night, PI-stained nuclei were analysed by flow cytometry (FACSCalibur, BD Biosciences).

7.2 BrdU/7-amino-actinomycin D co-staining.

This two-colour flow cytometric analysis permits the enumeration and characterisation of cells that are actively synthesising DNA (BrdU incorporation, FITC labeled) in terms of their cell cycle position (defined by 7-AAD staining intensities). 7-amino-actinomycin D (7-AAD) is used for determination of DNA content instead of PI in this experiment as the light emitted by 7-AAD and FITC does not overlap and can thus be analysed in distinct channels.

Cells were seeded at a density of 80,000 cells/well in 6-well plates and made quiescent by serum withdrawal for 4 days. Cells were stimulated by addition of 10% calf serum. 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 S-phase arrest. Subsequently, aphidicolin was removed by washing cells once with PBS before adding fresh growth medium containing 2 µM aphidicolin, 100 µM RV or vehicle for another 4 hours. The last 30 minutes, cells

were pulse labelled with 10 µM BrdU, harvested and processed as described by the manufacturer (BrdU Flow Kit, BD Biosciences). Briefly, cells were fixed and permeabilised using buffers provided by the kit, followed by treatment with DNAse to expose BrdU epitopes. These were labelled using FITC conjugated anti-BrdU specific antibodies. Additionally, DNA was stained with 7-AAD. Analysis was performed by flow cytometry (FACSCalibur, BD Biosciences, Germany).

To determine whether resveratrol induced cell cycle block is reversible, cells were seeded at a density of 80,000 cells/well in 6-well plates. After serum starvation for 2 days, cell cycle arrest was induced by treatment with 100 µM resveratrol in growth medium for 19 hours. Resveratrol was removed by washing cells once with PBS. Afterwards, fresh growth medium with or without 100 µM Resveratrol was added and cells were grown for another 4 hours. During the last 30 min, cells were pulse labelled with 10 µM BrdU and processed as described above.

8 Statistical analysis

All experiments were performed at least three times. Results are expressed as mean ±S.E. Statistical analysis was performed by ANOVA followed by a Dunnett multiple comparison test or a one-sample t test using GraphPad Prism version 3.02 for Windows (GraphPad Software Inc., San Diego, USA). P values < 0.05 were considered significant.

D. Results

1 Characterisation of VSMCs

VSMCs were isolated from rat thoracic aortas as described in the Materials and Methods section. Since this thesis was the first to deal with VSMCs in this group, evaluation of the protocol used for isolation was of considerable importance. Fig. 9 shows that isolated cells stained with FITC-labelled anti-α-smooth muscle actin exhibited a bright green fluorescence, identifying them as VSMCs. When Human Umbilical Vein Endothelial cells (HUVEC), Raw 264.7 cells and L929 murine fibroblasts were used in the same experimental settings, no staining was observed (data not shown), arguing for sufficient specificity of the antibody.

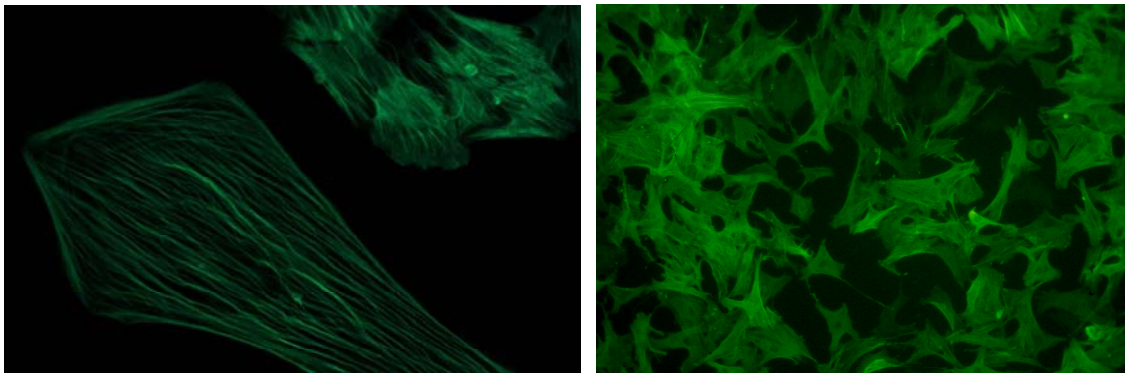

Fig. 9. VSMCs stained with FITC-labelled anti-α-smooth muscle actin. Left panel shows a confocal image of one stained cell (400x), right panel shows a fluorescence microscopic image of a VSMC culture (100x).

2 Influence of resveratrol on Ang II- and EGF-treated VSMCs

2.1 VSMC hypertrophy

2.1.1 Ang II induces hypertrophy but not hyperplasia in VSMCs

VSMC hypertrophy is an important feature in the development of cardiovascular disease, and Ang II is a pivotal stimulus in this process. Consistent with results reported by others (Takahashi *et al.*, 1997; Schmidt-Ott *et al.*, 2000), Fig. 10 shows that Ang II induces protein synthesis but not proliferation in VSMCs.

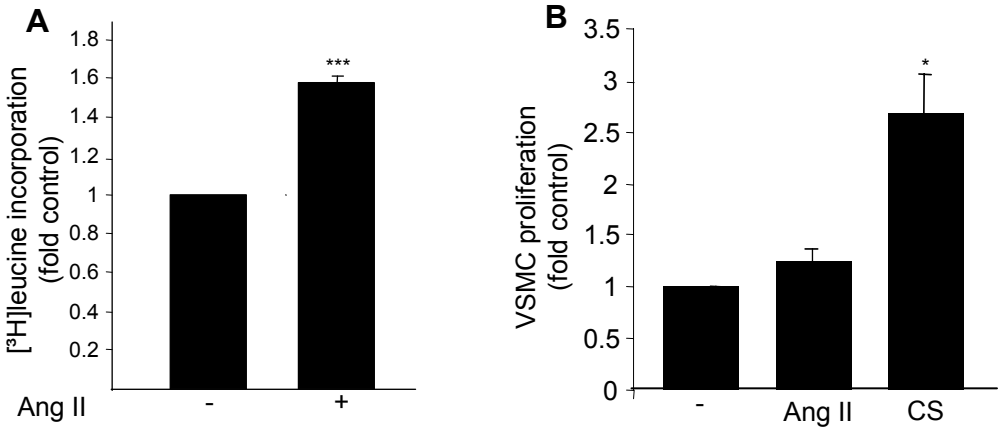

*Fig. 10. Ang II induces hypertrophy but not hyperplasia in VSMCs. A, VSMCs were serum-starved by incubation in DMEM containing 0.1% calf serum for 48 hours before treatment with 1 µCi/ml [³H]leucine in the presence (+) or absence (-) of 100 nM Ang II. 24 hours later cells were harvested and [³H]leucine incorporation was assessed as described in Materials and Methods. B, cells were serum-starved over night in DMEM containing 0.1% calf serum, left untreated (-) or stimulated with 100 nM Ang II or 10% calf serum for 24 hours, harvested and counted in a Fuchs-Rosenthal chamber. Graph shows averaged data from three independent experiments performed in triplicate. ***p<0.001; *p<0.05 (one-sample t test)*

2.1.2 Resveratrol inhibits Ang II-induced VSMC hypertrophy

To test whether resveratrol is able to block the increase in protein synthesis observed upon Ang II stimulation, cells were pre-incubated with different concentrations of resveratrol 30 minutes prior to stimulation with Ang II. The MEK 1 inhibitor PD98,059 and wortmannin, both substances previously described to inhibit Ang II-induced hypertrophy (Servant *et al.*, 1996; Ushio-Fukai *et al.*, 1999), were used as positive controls.

Fig. 11A clearly shows a concentration dependent decrease in Ang II-induced [³H]leucine incorporation in resveratrol treated cells that reaches significance at 25 µM resveratrol. Of note, both resveratrol and wortmannin also reduced basal levels of protein synthesis (Fig. 11B).

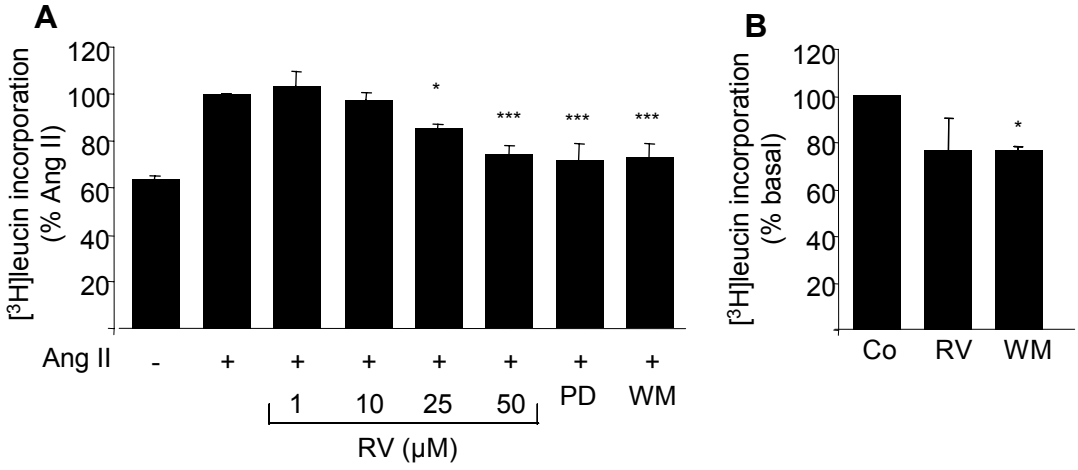

*Fig. 11. Resveratrol inhibits basal and Ang II-induced increase in [³H]leucine incorporation. A, VSMCs were serum-starved in DMEM supplemented with 0.1% calf serum for 48 hours, pre-treated with vehicle, the indicated concentrations of resveratrol (RV), 20 µM PD98,059 (PD) or 50 nM wortmannin (WM) for 30 minutes and subsequently stimulated with 100 nM Ang II for 24 hours. B, VSMCs were serum-starved for 48 hours in DMEM supplemented with 0.1% calf serum and treated with vehicle, 50 µM RV or 50 nM WM for 24 hours. Cells were processed as described in Materials and Methods. * p<0.05; ***p<0.001 (ANOVA/Dunnett and one-sample t test)*

2.2 Phosphorylation of Akt, p38, Erk 1/2 and p70^{S6k}

2.2.1 Ang II induces phosphorylation of Akt, p38, Erk 1/2 and p70^{S6k}

The exact signalling mechanisms leading to VSMC hypertrophy are only partially understood. For Ang II-induced hypertrophy in VSMCs, the mitogen-activated protein kinase (MAPK) Erk 1/2, the MAPK p38, the serine/threonine kinase Akt as well as p70^{S6k} have been implicated (Ushio-Fukai *et al.*, 1998; Servant *et al.*, 1996; Ushio-Fukai *et al.*, 1999). It is generally accepted that phosphorylation correlates with the activity of these kinases (Ushio-Fukai *et al.*, 1999; Ushio-Fukai *et al.*, 1998; Frank *et al.*, 2000; Weng *et al.*, 1998). Therefore, we performed Western blot analysis using antibodies against phospho-kinases.

As depicted in Fig. 12, Ang II treatment led to a rapid increase in phosphorylation of the above-mentioned kinases.

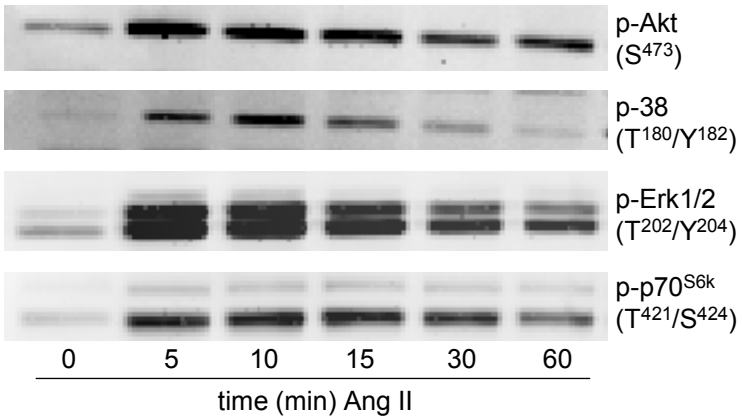

Fig. 12. Time course of Ang II-induced phosphorylation of Akt, p38 MAPK, Erk 1/2, and p70^{S6k}. VSMCs in 0.1% calf serum were treated with 100 nM Ang II for the indicated times. Western blot analysis was performed as described in Materials and Methods. Panels show one representative Western blot out of three.

2.2.2 Resveratrol inhibits phosphorylation of Akt, Erk 1/2 and p70^{S6k}

We next examined whether resveratrol affects activation of these kinases. As shown in Fig. 13, resveratrol inhibited Ang II-induced Akt and p70^{S6k} phosphorylation. Reduction of Ang II-stimulated Erk 1/2 phosphorylation was less pronounced, while p38 MAPK phosphorylation was not affected at all.

It is important to note that resveratrol alone had no effect on phosphorylation of these kinases (Fig. 14).

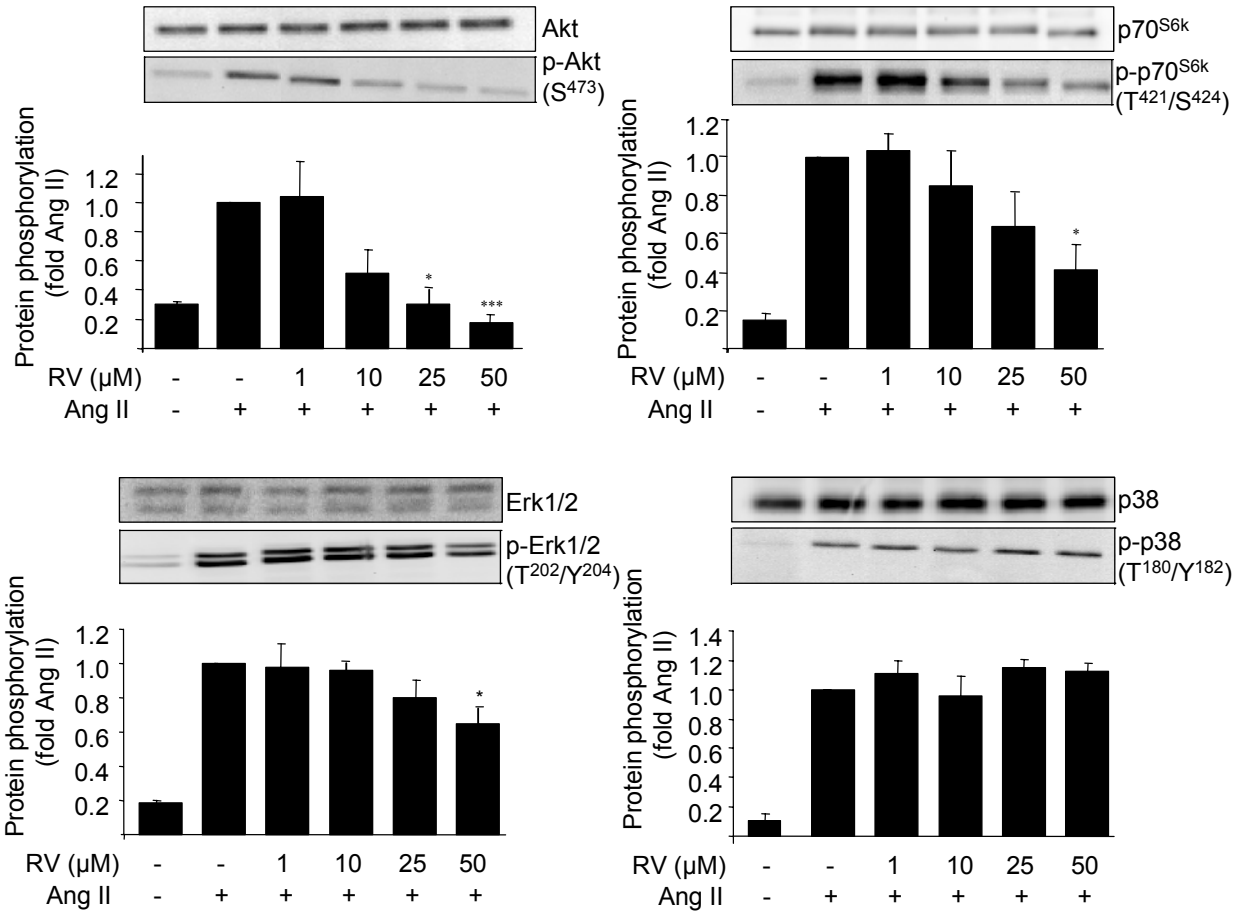

*Fig. 13. Effect of resveratrol on Ang II-induced Akt, p70^{S6k}, Erk 1/2 and p38 phosphorylation. VSMCs in 0.1% calf serum were treated with resveratrol (RV) in the indicated concentrations or vehicle (-) for 30 minutes and subsequently stimulated with (+) or without (-) 100 nM Ang II for 10 minutes. Top panels show representative Western blots depicting total levels of kinases. Lower panels show levels of phosphorylated kinases. Graphs at the bottom represent signal intensities obtained by densitometric analysis of three immunoblots. *p<0.05; ***p<0.001 (ANOVA/Dunnett)*

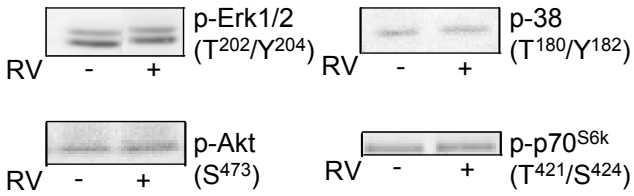

Fig. 14. Resveratrol does not enhance phosphorylation of Erk 1/2, p38, Akt and p70^{S6k}. VSMCs were serum-starved over night and treated with (+) or without (-) 50 µM resveratrol for 40 minutes. Panels show one representative Western blot out of two depicting levels of phosphorylated kinases.

2.3 Protein phosphatase 2A

Resveratrol was most effective in reducing Akt kinase activity, so we focused on this kinase in further experiments. To gain insight into whether resveratrol inhibits Akt phosphorylation by activation of phosphatases, we examined whether okadaic acid, an inhibitor of the phosphatase PP2A (Millward *et al.*, 1999), is able to override the observed resveratrol effect. Wortmannin, known to work via inhibition of phosphoinositide 3-kinase (PI3-k) rather than activation of phosphatases, was used as control.

Western blot analysis clearly shows that both resveratrol and the PI3-k inhibitor wortmannin reduced Akt phosphorylation and that this effect was not abrogated by okadaic acid, even though, on first sight, the extent of inhibition was reduced (Fig. 15A).

The slight increase in Akt phosphorylation in the presence of okadaic acid was, however, most likely caused by okadaic acid itsself, because okadaic acid alone enhanced phospho-Akt levels to a similar extent (Fig. 15B).

Thus, resveratrol does not seem to act *via* activation of type 2A phosphatases.

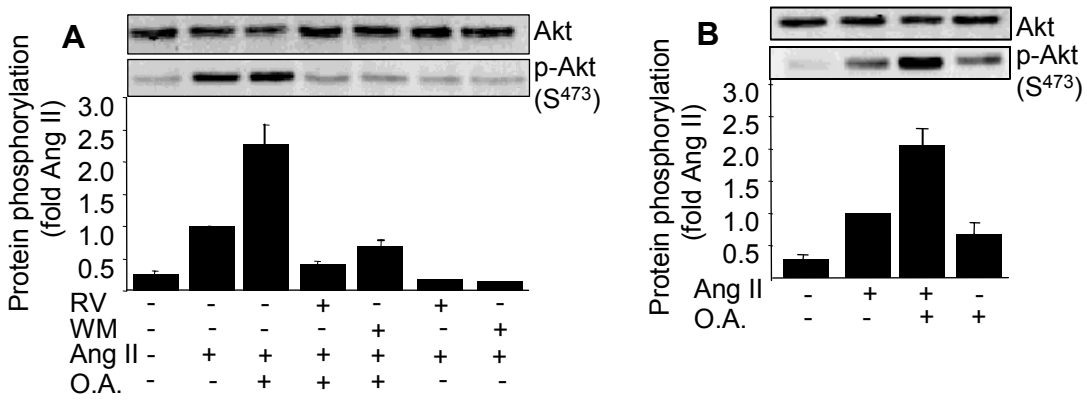

Fig. 15. Resveratrol does not activate okadaic acid-inhibitable phosphatases. A, okadaic acid does not override the effects of resveratrol and wortmannin on Akt phosphorylation. VSMCs in 0.1% calf serum were preincubated with 50 µM resveratrol (RV), 50 nM wortmannin (WM) or vehicle (-) for 15 minutes, subsequently treated with (+) or without (-)1 µM okadaic acid (O.A.) for an additional 30 minutes and finally stimulated with (+) or without (-)100 nM Ang II for 10 minutes. B, okadaic acid itself enhances phospho-Akt levels. VSMCs in 0.1% calf serum were treated with (+) or without (-) 1 µM O.A. for 30 minutes before stimulation with (+) without (-) Ang II. Top panels show representative Western blots for total levels of Akt kinase. Lower panels depict levels of phosphorylated Akt. Graphs at the bottom show mean signal intensities obtained by densitometric analysis of four immunoblots.

2.4 Phosphorylation of PI 3-kinase p85 subunit

We next investigated pathways *upstream* of Akt kinase. PI3-k is essential for Akt activation and can therefore be considered a possible target (Datta *et al.*, 1999). Saward and Zahradka have demonstrated that Ang II stimulates tyrosine phosphorylation of the regulatory p85 subunit of PI3-k and, accordingly, PI3-k activity in porcine coronary artery VSMCs (Saward and Zahradka, 1997).

As shown in Fig. 16A, tyrosine phosphorylation of the p85 subunit peaked at 5 minutes of Ang II stimulation. This effect was dramatically reduced by pre-treating the cells with resveratrol (Fig. 16B), suggesting that resveratrol indeed acts *upstream* of Akt kinase by inhibiting PI3-k phosphorylation and activation.

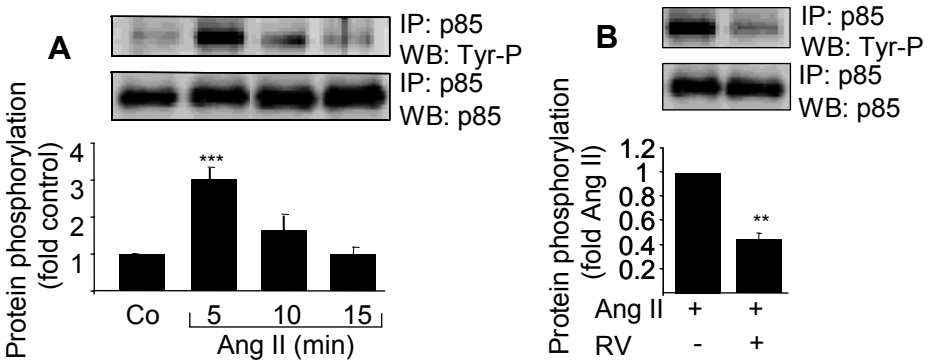

*Fig. 16. Resveratrol inhibits tyrosine phosphorylation of the p85 PI 3-k subunit. A, time course for p85 phosphorylation in response to Ang II. VSMCs in 0.1% calf serum were stimulated with Ang II for the indicated times. B, resveratrol prevents Ang II-induced p85 phosphorylation. VSMCs in 0.1% calf serum were pretreated with 50 µM resveratrol (RV) or vehicle for 30 minutes before stimulation with Ang II for 5 minutes. Immunoprecipitation (IP) was performed as described in Materials and Methods. Immunoprecipitated proteins were analysed by Western blot (WB) using an anti-phospho-tyrosine antibody (top panels) or an anti-p85 antibody (lower panels). Graphs at the bottom show mean signal intensities obtained by densitometric analysis of three immunoblots. ***p<0.001 (A, ANOVA/Dunnett; B, one-sample t-test)*

2.5 Calf serum-induced Akt activation

In order to investigate whether resveratrol directly inhibits PI3-k activity, we performed additional experiments using calf serum as a stimulus, since serum-induced Akt activation has been shown to be PI3-k dependent as well (Jung *et al.*, 2000; Andjelkovic *et al.*, 1996).

As a positive control, we used wortmannin, known to directly inhibit PI3-k by covalent modification of the enzyme`s catalytic subunit (Wymann *et al.*, 1996).

Interestingly, in striking contrast to wortmannin, resveratrol did not inhibit serum-induced Akt phosphorylation at a concentration of 50 µM (Fig. 17). Consequently, resveratrol cannot be considered an inhibitor of PI 3-k itself.

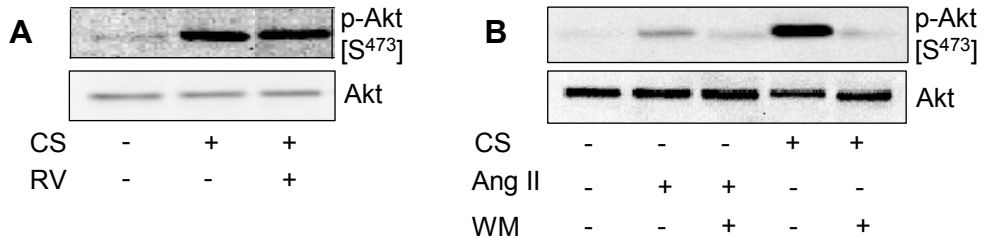

Fig. 17. Resveratrol and wortmannin differently affect calf serum- and Ang II-induced Akt phosphorylation. A, resveratrol does not inhibit calf serum-induced Akt phosphorylation. Cells were treated with vehicle (-) or 50 µM resveratrol (RV) for 30 minutes before stimulation with (+) or without (-) 10% calf serum (CS) for 30 minutes. B, wortmannin inhibits both Ang II and calf serum-induced Akt phosphorylation. Quiescent VSMCs were treated with vehicle or 50 nM wortmannin (WM) for 30 minutes before stimulation with (+) or without (-) Ang II (10 minutes) or CS (30 minutes). Lysates were prepared as described in Materials and Methods. Bottom panels show representative Western blots for total levels of Akt kinase. Top panels depict levels of phosphorylated Akt.

2.6 EGF-R transactivation

2.6.1 Ang II-induced transactivation of the EGF-R is involved in Akt activation

In recent years, it has become evident that G-protein coupled receptors transactivate receptor tyrosine kinases, which then serve as scaffold for various signalling molecules and mediate further signal transduction. For the AT_1-R, transactivation of the EGF-R has been demonstrated (Kalmes *et al.*, 2001).

Consistently, Ang II caused a rapid increase in EGF-R phosphorylation in our cells that peaked at 2 minutes and remained above baseline for 10 minutes (Fig. 18).

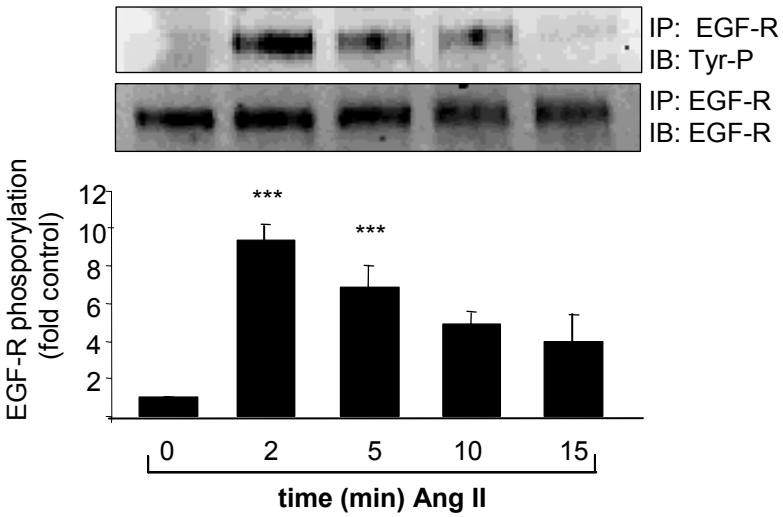

*Fig. 18. Time course of Ang II-induced EGF-R phosphorylation in VSMCs. VSMCs were stimulated with 100 nM Ang II for the indicated times. Lysates were immunoprecipitated (IP) with anti-EGF-R antibody, followed by immunoblotting (IB) with anti-phospho-tyrosine (Tyr-P, top panel) or anti-EGF-R antibody (lower panel). Graph at the bottom shows averaged data obtained by densitometric analysis of three immunoblots, expressed as fold increase in phosphorylation over control. ***p<0.001 (ANOVA/Dunnett)*

Previous reports present evidence that activation of Akt kinase by Ang II in VSMCs occurs in an EGF-R dependent manner (Ushio-Fukai *et al.*, 2001a; Eguchi *et al.*, 1999b). In order to verify that transactivation of EGF-R is implicated in Akt activation in our cells, we treated VSMCs with AG 1478, an inhibitor of intrinsic EGF-R kinase activity, prior to stimulation with Ang II.

AG 1478 substantially decreased Akt phosphorylation (Fig. 19) but failed to completely abolish the signal. These data indicate that EGF-R is indeed important for Ang II-induced Akt phosphorylation, although parallel pathways should as well be taken into account.

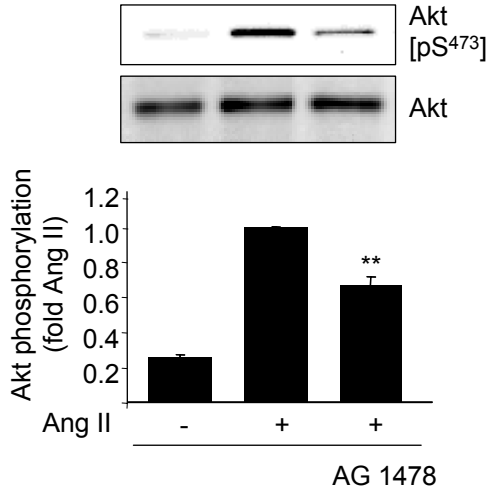

*Fig. 19. AG 1478 inhibits Ang II-induced Akt phosphorylation. VSMCs were pre-treated with vehicle or 250 nM AG 1478 before treatment with (+) or without (-) 100 nM Ang II for 10 minutes. Lysates were prepared as described in Materials and Methods. Bottom panel shows a representative Western blot for total levels of Akt kinase. Top panel depicts the level of phosphorylated Akt. Graph at the bottom shows averaged data obtained by densitometric analysis of three independent experiments, expressed as fold inhibition of Ang II-induced Akt phosphorylation. **p<0.01 (one-sample t test)*

2.6.2 Resveratrol does not attenuate EGF-R transactivation

Since EGF-R transactivation is indeed involved in Akt phosphorylation, we tested the ability of resveratrol to interfere with EGF-R transactivation. Agents previously described to act as inhibitors of this process (the antioxidant *N*-acetyl-cysteine (NAC), PP1, an inhibitor of c-Src kinase, and AG 1478) were used as positive controls.

Fig. 20 clearly shows that resveratrol, in contrast to NAC, PP1 and AG 1478, does not influence EGF-R transactivation.

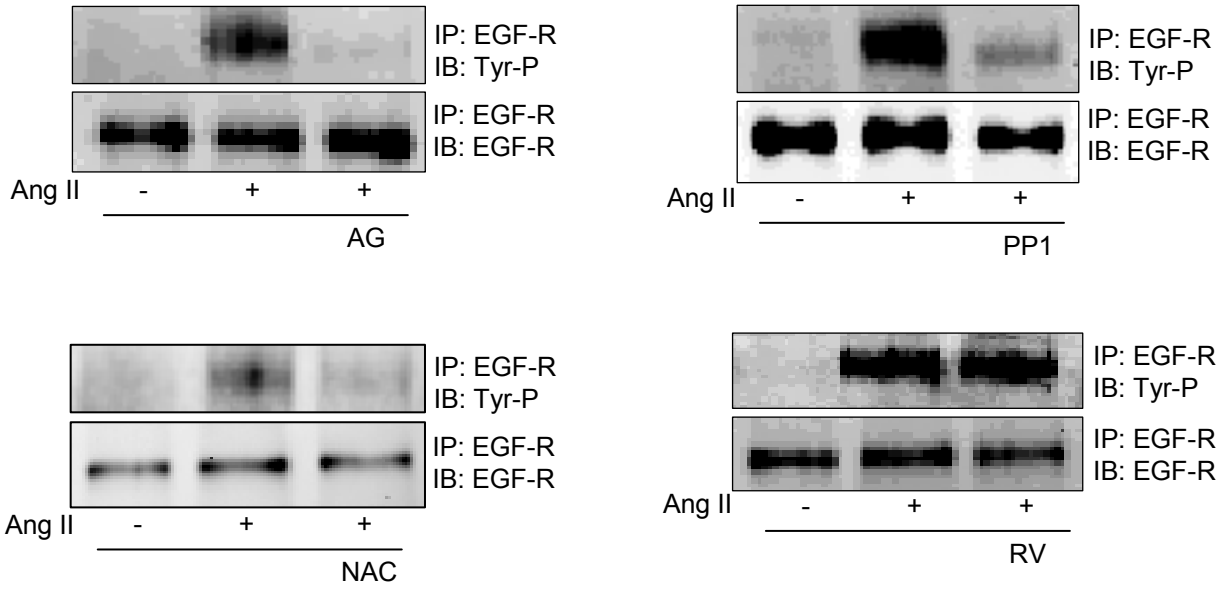

Fig. 20. AG 1478, PP1 and NAC but not resveratrol attenuate Ang II-induced EGF-R phosphorylation. VSMCs were pre-incubated with 250 nM AG 1478 (AG), 20 µM PP1, 10 mM NAC or 50 µM resveratrol (RV) for 30 minutes before treatment with (+) or without (-) 100 nM Ang II for 2 minutes. Lysates were immunoprecipitated (IP) with anti-EGF-R antibody, followed by immunoblotting (IB) with anti-phospho-tyrosine (Tyr-P, top panel) or anti-EGF-R antibody (bottom panel). Panels show one representative Western blot out of three.

2.7 EGF-induced Akt and Erk 1/2 activation

EGF-R transactivation is not impeded by resveratrol. We, therefore, postulated that resveratrol must act downstream of EGF-R.

2.7.1 EGF induces Akt and Erk 1/2 activation

EGF rapidly stimulated Akt phosphorylation with a peak at 2 minutes. Erk 1/2 phosphorylation occurred quickly but was sustained for the time period investigated (Fig. 21).

Consequently, we investigated whether EGF-stimulated Akt and Erk 1/2 activation is sensitive to resveratrol.

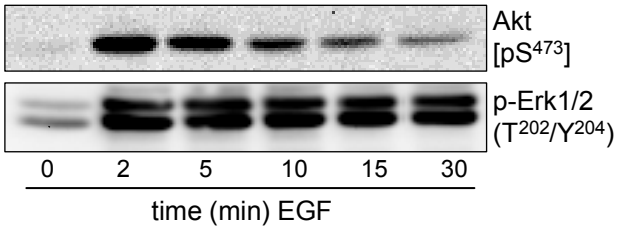

Fig. 21. Time course of EGF-induced Akt and Erk 1/2 phosphorylation. VSMCs were treated with (+) or without (-) 100 ng/ml EGF for the indicated times. Lysates were prepared as described in Materials and Methods. Panels show a representative Western blot out of three for levels of phosphorylated Akt or Erk 1/2.

2.7.2 Resveratrol inhibits EGF-induced Akt phosphorylation

Fig. 22 shows that resveratrol as well as PP1, AG 1478 and wortmannin strongly inhibited EGF-induced Akt phosphorylation, suggesting that resveratrol indeed acts downstream of EGF-R in regard of Akt activation. Interestingly, phosphorylation of Erk 1/2 was not significantly altered by resveratrol after EGF stimulation (Fig. 23).

These results suggest that resveratrol predominantly interferes with the Akt and not the Erk 1/2 pathway upon stimulation with Akt or EGF.

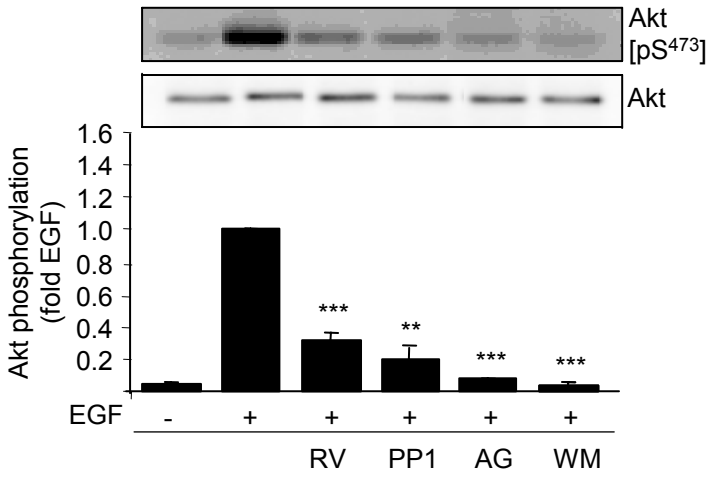

*Fig. 22. Effect of resveratrol, AG 1478, PP1 and wortmannin on EGF-induced Akt phosphorylation. VSMCs were pre-incubated with vehicle, 50 μM resveratrol (RV), 20 μM PP1, 250 nM AG 1478 (AG), or 50 nM wortmannin (WM) for 30 minutes before treatment with (+) or without (-) 100 ng/ml EGF for 4 minutes. Lysates were prepared as described in Materials and Methods. Lower panel shows a representative Western blot for total levels of Akt kinase. Top panel depicts the level of phosphorylated Akt. Graph at the bottom shows averaged data obtained by densitometric analysis of four independent experiments, expressed as fold inhibition of EGF-induced phosphorylation. **p<0.01, ***p<0.001 (one-sample t test)*

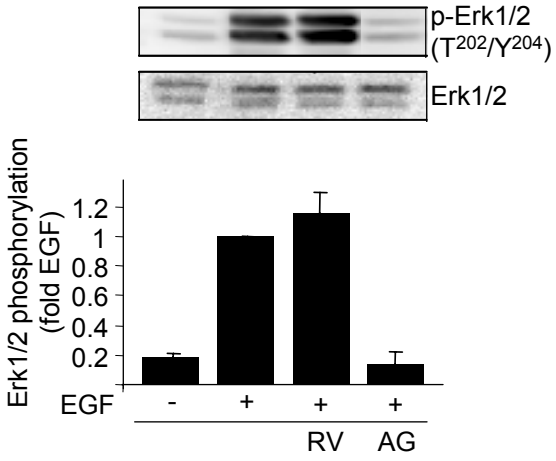

Fig. 23. Effect of resveratrol and AG 1478 on EGF-induced Erk 1/2 phosphorylation. VSMC in DMEM supplemented with 0.1% calf serum were pre-treated with vehicle, 50 μM resveratrol (RV) or 250 nM AG 1478 (AG) for 30 minutes before treatment with 100 ng/ml EGF for 4 minutes. The top panel shows a representative Western blot for phosphorylated Erk 1/2 kinase. The lower panel depicts total levels of Erk 1/2 kinase. Graph represents averaged data obtained by densitometric analysis of three (or two, for AG) independent experiments.

2.8 c-Src kinase

PP1 was able to inhibit EGF-mediated Akt phosphorylation, suggesting that c-Src plays a role in signal transduction from the EGF-R to Akt. Resveratrol, on the other hand, has been described to function as an inhibitor of c-Src kinase in Hela cells (Yu *et al.*, 2001). Accordingly, we investigated a possible impact of resveratrol on c-Src kinase.

2.8.1 Ang II induces c-Src phosphorylation.

We first performed a time course for Ang II-induced phosphorylation of sites important for regulation of c-Src kinase function.

As shown in Fig. 24, Ang II induced a rapid increase in both tyrosine^{215} and tyrosine^{418} phosphorylation.

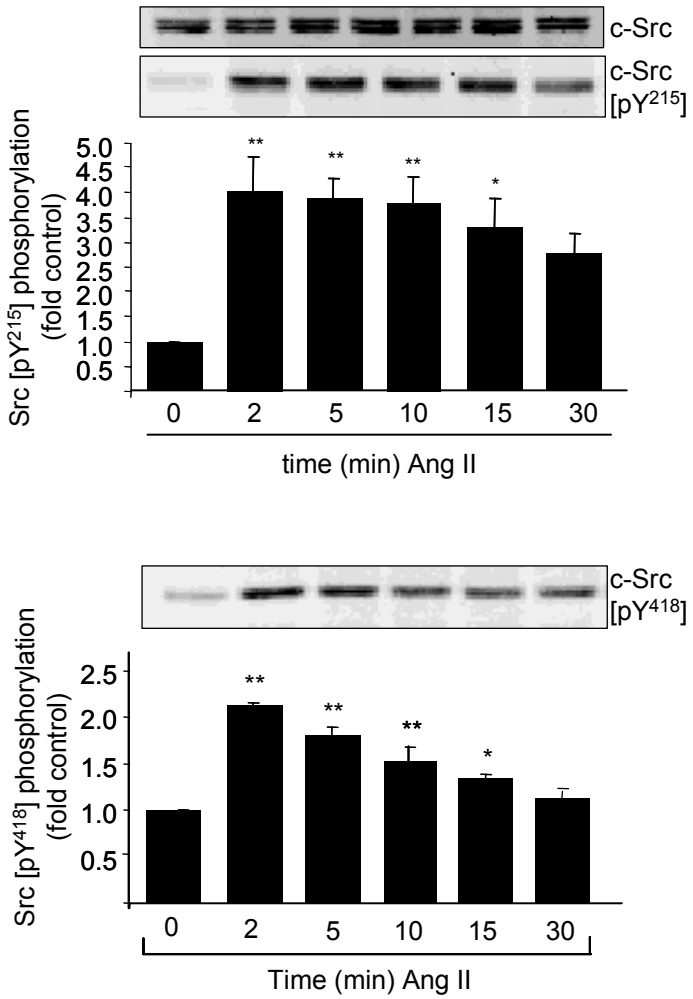

*Fig. 24. Time course of Ang II-induced phosphorylation of c-Src kinase. VSMCs were stimulated with 100 nM Ang II for the indicated times. Lysates were immunoblotted with anti-phospho-c-Src (pY^{215} or pY^{418}, lower panels) or anti-c-Src antibody (top panel). Graphs show averaged data obtained from densitometric analysis of three independent experiments, expressed as fold increase over control. *p<0.05, **p<0.01 (ANOVA/Dunnett)*

2.8.2 Effect of resveratrol on c-Src phosporylation

We next tested a possible influence of resveratrol-treatment on phosphorylation of these sites. However, although resveratrol did decrease Ang II-induced phosphorylation of tyrosine^{215}, there was no inhibition of tyrosine^{418} phosphorylation (Fig. 25).

Considering that tyrosine^{418} is subject to autophosphorylation and phosphorylation is a prerequisite for complete activation (Martin, 2001; Tatosyan and Mizenina, 2000), this result strongly suggests that resveratrol has no influence on Ang II-induced c-Src activity.

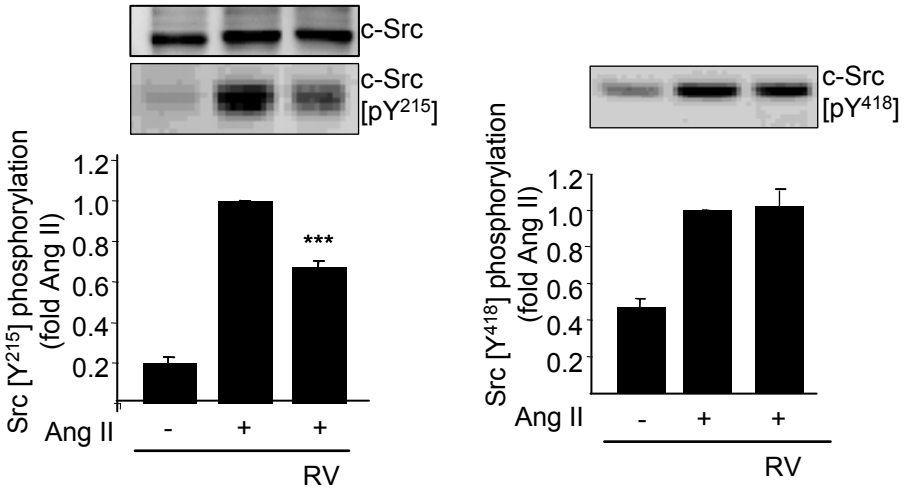

*Fig. 25. Effect of resveratrol on Ang II-induced phosphorylation of c-Src. VSMCs were pre-incubated with vehicle (-) or 50 µM resveratrol (RV) for 30 minutes prior to stimulation with 100 nM Ang II for 3 minutes. Lysates were immunoblotted with anti-phospho-c-Src (pY^{215}, left panel, or pY^{418}, right panel) or anti-c-Src antibody (top panel on the left). Graphs show averaged data obtained from densitometric analysis of four independent experiments, expressed as inhibition of Ang II-induced phosphorylation. ***p<0.001 (one-sample t test)*

2.8.3 EGF-R tyrosine^{845} phosphorylation

To further corroborate this hypothesis, we tested whether resveratrol interferes with phosphorylation of EGF-R tyrosin^{845}, a target of c-Src kinase (Sato *et al.*, 1995;Biscardi *et al.*, 1999). Tyrosine^{845} is phosphorylated upon Ang II stimulation ((Ushio-Fukai *et al.*, 2001b) and data not shown), however, the signal was much more pronounced after EGF treatment. Therefore, in the experiments described below, EGF was used as a stimulus.

EGF-R tyrosine^{845} was rapidly induced upon EGF stimulation (Fig. 26), and phosphorylation was completely abolished by pre-treatment of cells with AG 1478 (Fig. 27). As expected, resveratrol did not decrease EGF-R phosphorylation at this site (Fig. 27).

Taken together, these results suggest that resveratrol does not attenuate c-Src kinase activity.

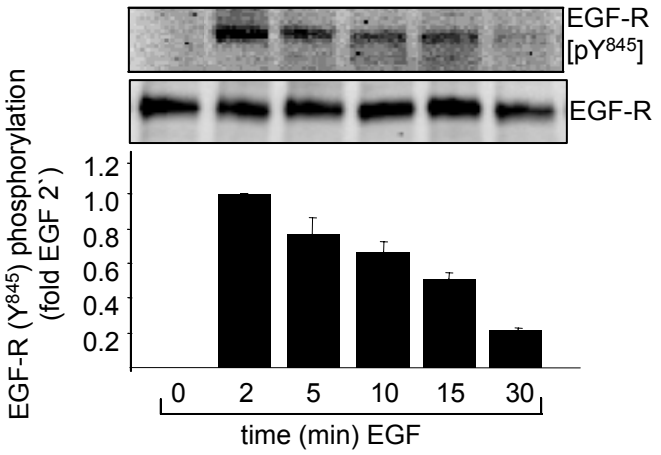

Fig. 26. Time course of EGF-induced EGF-R phosphorylation. VSMCs were treated with 100 ng/ml EGF for the indicated times. Lysates were immunoblotted with anti-phospho-EGF-R (pY^{845}, top panel) or anti-EGF-R antibody (lower panel). Graph shows averaged data obtained by densitometric analysis of three immunoblots.

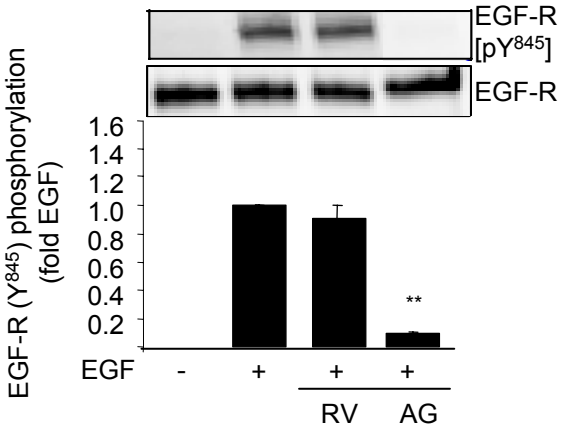

*Fig. 27. Resveratrol does not inhibit EGF-induced phosphorylation of EGF-R. VSMCs were treated with vehicle, 50 µM resveratrol (RV) or 250 nM AG 1478 (AG) for 30 minutes prior to stimulation with 100 ng/ml EGF for 2 minutes. Graph shows averaged data obtained from densitometric analysis of four (RV) or two (AG) independent experiments. **p<0.01 (one-sample t test)*

3 Influence of resveratrol on calf serum-treated VSMCs

3.1 Cell cycle progression

3.1.1 Calf serum-induced cell cycle progression

Flow cytometric cell cycle analysis revealed that VSMCs require about 10-12 hours to reach S-phase and 22-24 hours to complete one cell cycle (Fig. 28).

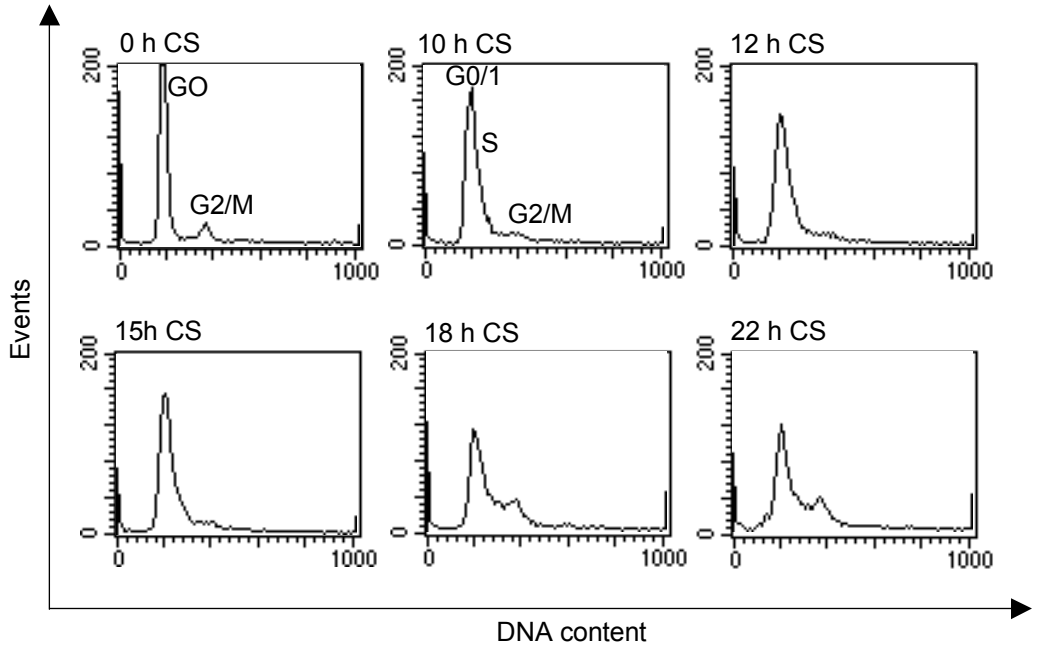

Fig. 28. Cell cycle progression of VSMCs upon calf serum stimulation. Quiescent VSMCs were stimulated with 10% calf serum for the indicated times. Cells were harvested and processed as described in Materials and Methods. Images show one representative experiment out of three.

3.1.2 Resveratrol inhibits VSMC proliferation

Previous studies demonstrate an antiproliferative effect of resveratrol in various cancer cell lines (Ahmad *et al.*, 2001; Sgambato *et al.*, 2001; Hsieh and Wu, 1999) as well as in cardiovascular cells (Zou *et al.*, 1999; Hsieh *et al.*, 1999).

Consistent with these results, we observed a dose-dependent reduction of calf serum-induced proliferation in VSMCs treated with 1-25 µM resveratrol (Fig. 29).

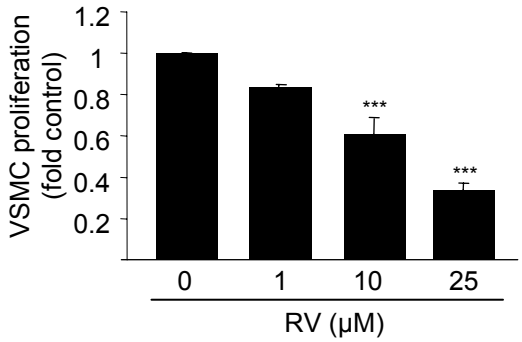

*Fig. 29. Resveratrol inhibits VSMC proliferation. VSMCs were serum-starved for 2 days before stimulation with the indicated concentrations of resveratrol (RV) in the presence of 10% calf serum. Fresh growth medium and resveratrol was added every day. On day four, cells were harvested and counted in a Fuchs-Rosenthal chamber. Graph shows averaged data from three independent experiments performed in triplicate normalised to non treated cells. ***p<0.001 (ANOVA/Dunnett)*

In order to elucidate the exact mechanisms underlying the antimitogenic effect of resveratrol in VSMCs, flow cytometric cell cycle analysis of VSMCs treated with various concentrations of resveratrol for 22 hours were performed.

The data obtained suggest a dose-dependent accumulation of resveratrol-treated cells at the G1/S-interphase, with 100 µM resveratrol leading to a complete block in cell cycle progression (Fig. 30).

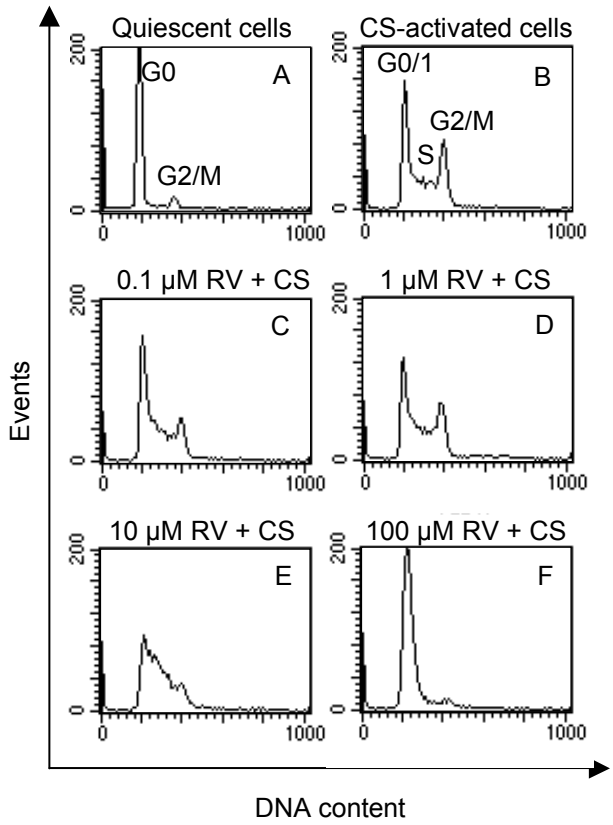

Fig. 30. Resveratrol-induced changes in cell cycle distribution of calf serum stimulated cells. Cells were serum-starved for four days and pre-incubated with various concentrations of resveratrol (RV) for 30 minutes (A, 0 µM; B, 0 µM; C, 0.1 µM; D, 1 µM; E, 10 µM; F, 100 µM). Subsequently, cells were stimulated with (B-F) or without (A) 10% calf serum (CS) for 22 hours. Cells were harvested and nuclei stained with PI as described in Materials and Methods. Data show one representative experiment out of three.

3.1.3 Resveratrol-induced cell cycle arrest is reversible

The DNA replication block depicted in Fig. 30 was, however, reversible, as cells arrested by resveratrol treatment were still able to cycle through S-phase when resveratrol was removed (Fig. 31).

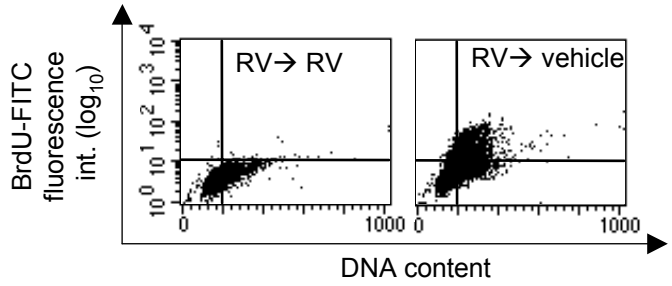

Fig. 31. Resveratrol-induced cell cycle arrest is reversible. Quiescent VSMCs were treated with 100 µM resveratrol (RV) in growth medium for 19 hours. Medium was changed and cells were treated with (RV→RV) or without (RV→vehicle) resveratrol for four hours in the presence of 10% calf serum. During the last 30 minutes, cells were pulse-labelled with BrdU and processed as described in Materials and Methods. Images show results of one representative experiment out of three.

Of note, neither Hoechst, PI staining, a nucleosome ELISA, nor cell cycle analysis revealed cytotoxic effects at 100 µM resveratrol (data not shown and Fig. 30F); therefore, this concentration was used for all other cell cycle experiments.

3.2 Phosphorylation of Akt, Erk 1/2 and p70^{S6K}

It is widely accepted that mitogens promote cell cycle progression of quiescent cells *via* the Akt and the Erk 1/2 signalling cascades, as these are critically involved in accumulation of D-type cyclins in the G1-phase (Sherr and Roberts, 1999). p70^{S6k} is activated downstream of Akt and Erk 1/2 in VSMCs (Eguchi *et al.*, 1999b) and has been implicated in cell proliferation (Vinals *et al.*, 1999). We, therefore, hypothesised that inhibition of these kinases might contribute to the observed antimitogenic effect of resveratrol.

3.2.1 Calf serum induces phosphorylation of Akt, Erk 1/2 and p70^{S6k}

As shown in Fig. 32, serum stimulation led to a rapid increase in Akt, Erk 1/2 and p70^{S6k} phosphorylation that was sustained for several hours.

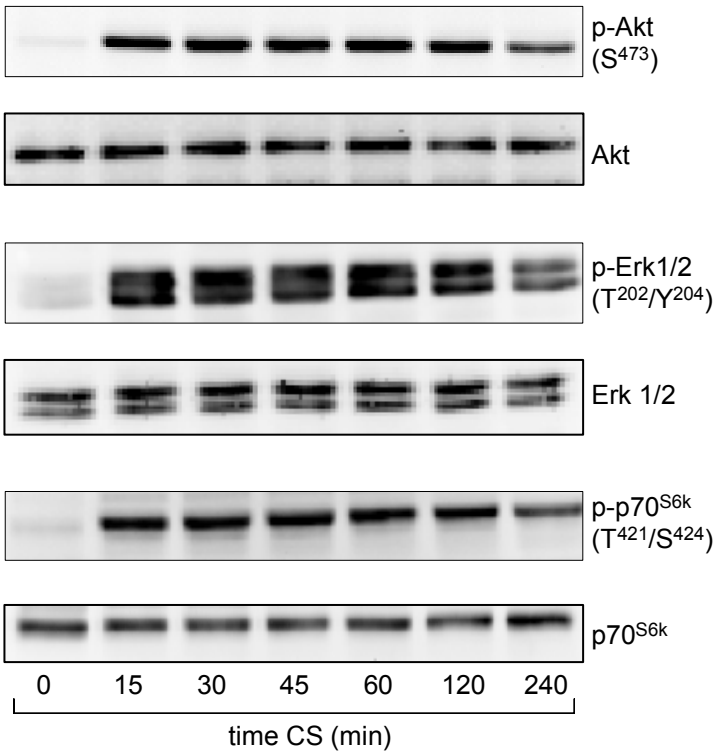

Fig. 32. Effect of calf serum stimulation on phosphorylation of Akt, Erk 1/2, and p70^{S6k}. Quiescent VSMCs were treated with 10% calf serum (CS) for the indicated times and lysates were prepared as described in Materials and Methods. Top panels show one representative Western blot out of three for phosphorylated kinases. Lower panels show total levels of kinases.

3.2.2 Effect of resveratrol on phosphorylation of Akt, Erk 1/2 and p70^{S6k}

However, resveratrol only weakly inhibited serum-mediated Akt and p70^{S6k} phosphorylation and failed to significantly alter Erk 1/2 phosphorylation (Fig. 33). Since resveratrol affected cell cycle progression in concentrations as low as 10 µM and arrest was complete at 100 µM, inhibition of these kinases is unlikely to primarily account for this effect.

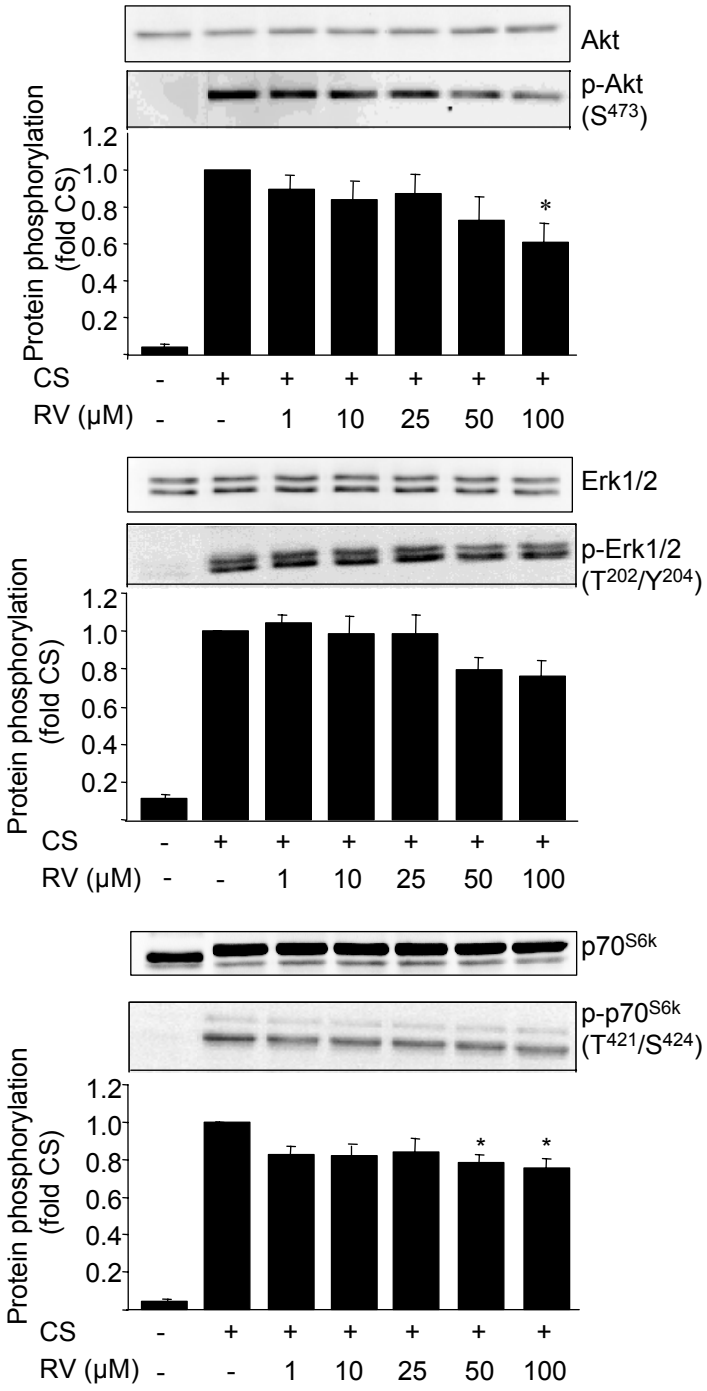

*Fig. 33. Effect of resveratrol on serum-induced phosphorylation of Akt, Erk 1/2 and p70^{S6k}. Serum-starved VSMCs were pre-incubated with resveratrol (RV) in the indicated concentrations for 30 minutes and subsequently stimulated with (+) or without (-) 10% calf serum (CS) for 30 minutes. Lower panels show a representative Western blot for phosphorylated kinases. Top panels show total levels of kinases. Graphs at the bottom represent averaged data obtained by densitometric analysis of three immunoblots and normalised to values of CS stimulated cells. *p<0.05 (ANOVA/Dunnett)*

3.3 Phosphorylation of Rb

In late G1-phase, cells reach the so-called restriction point. Beyond this point, cell cycle progression proceeds independent of growth factor stimulation (Sherr, 1996). Hypophosphorylated Rb binds members of the E2F family of transcription factors, and thus inhibits transcription of E2F-responsive genes necessary for cell cycle progression. Hyperphosphorylation of Rb by cyclin-dependent kinases leads to dissociation of Rb from promoter-bound E2F, allowing transcription of E2F regulated genes (Sherr, 1996; DePinho, 1998).

3.3.1 Calf serum increases Rb protein phosphorylation

Fig. 34A shows that calf serum leads to a continuous increase in Rb hyperphosphorylation over 22 hours. Of note, it has been reported that antimitogenic agents affecting the G1-phase, such as inhibitors of the PI3-k or the $p70^{S6k}$ pathway, keep Rb in the hypophosphorylated, active state (Vinals *et al.*, 1999). Consistently, the results of a control experiment presented in Fig. 34B show that wortmannin decreased phosphorylation levels of Rb.

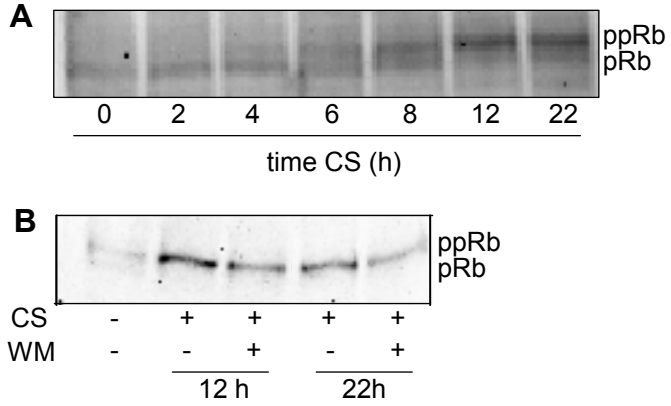

Fig. 34. Western blot analysis of Rb hyperphosphorylation. A, time course of Rb hyperphosphorylation in response to 10% calf serum. Quiescent VSMCs were stimulated with 10% calf serum (CS) for the indicated times. Panel depicts one representative Western blot out of three. B, wortmannin attenuates calf serum-induced Rb hyperphosphorylation. Quiescent VSMCs were pre-incubated with 50 nM wortmannin (WM) for 30 minutes prior to stimulation with 10% CS for the indicated times. Panel shows one representative Western blot out of two.

3.3.2 Resveratrol promotes Rb hyperphosphorylation

We next investigated the possible impact of resveratrol on calf serum-induced Rb hyperphosphorylation. Most interestingly, pre-treatment of cells with resveratrol did not prevent Rb hyperphosphorylation but resulted in a substantial increase in hyperphosphorylated Rb (Fig. 35).

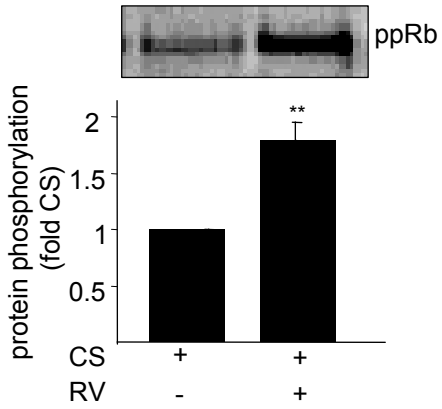

*Fig. 35. Resveratrol increases Rb hyperphosphorylation. Quiescent VSMCs were pre-treated with 100 µM resveratrol (RV) for 30 minutes prior to addition of 10% calf serum (CS) for 22 hours. Panel shows one representative Western for phosphorylated Rb. Graph shows averaged data obtained by densitometric analysis of five immunoblots.**p<0.01 (one-sample t test)*

3.4 $p21^{Cip1}$ and $p27^{Kip1}$ protein expression

Several studies have linked resveratrol with an upregulation of $p21^{Cip1}$ (Ahmad *et al.*, 2001;Hsieh *et al.*, 1999). Even though the Cip/Kip proteins are potent inhibitors of cyclin E- and A-dependent kinases, which are rate-limiting and essential for DNA replication, they act as positive regulators of cyclin D-dependent kinases that phosphorylate Rb (Sherr and Roberts, 1999). Therefore, it seemed possible that resveratrol may induce cell cycle arrest through upregulation of the cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$.

3.4.1 Calf serum regulation of $p21^{Cip1}$ and $p27^{Kip1}$ protein expression

Fig. 36 shows time courses of $p21^{Cip1}$ and $p27^{Kip1}$ in calf serum-stimulated VSMCs. As expected, $p21^{Cip1}$ levels were low in quiescent cells but up-regulated in late G1-phase. In contrast, $p27^{Kip1}$ levels decreased over time upon stimulation with calf serum as reported before (Sherr and Roberts, 1999).

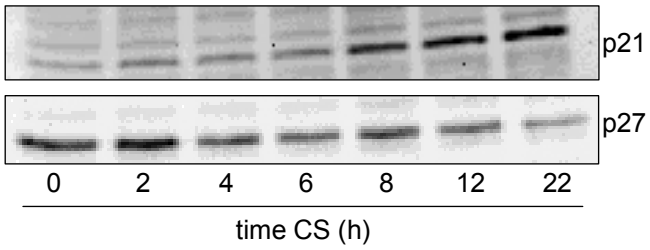

Fig. 36. Time course of $p21^{Cip1}$ and $p27^{Kip1}$ protein expression in response to calf serum. Quiescent VSMCs were stimulated with 10% calf serum (CS) for the indicated times. Panels show one representative Western blot out of three.

3.4.2 Resveratrol reduces levels of $p21^{Cip1}$and $p27^{Kip1}$

Resveratrol, however, in contrast to results from other groups using different cell systems, neither induced $p21^{Cip1}$ nor $p27^{Kip1}$ protein expression. Quite the contrary, levels of these cyclin-dependent kinase inhibitors were lower in resveratrol-treated VSMCs (Fig. 37).

Taken together, these results suggest that resveratrol does not affect cell cycle progression in the G1-phase.

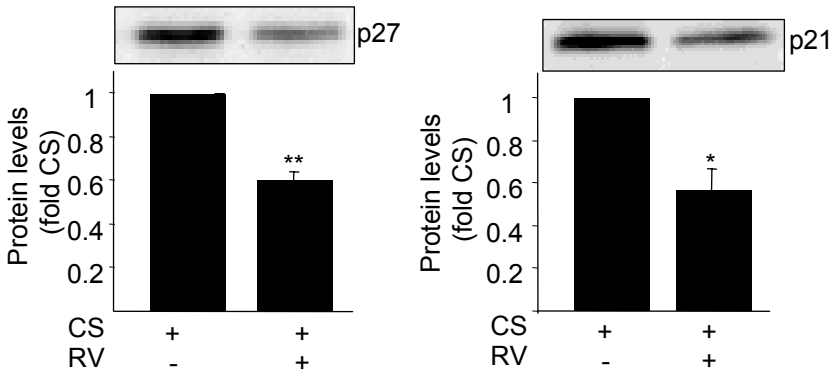

*Fig. 37. Effect of resveratrol on $p21^{Cip1}$ and $p27^{Kip1}$ protein expression in calf serum-stimulated cells. Quiescent VSMCs were pre-incubated with 100 µM resveratrol (RV) for 30 minutes before stimulation with 10% calf serum (CS) for 22 hours. Blots show one representative Western blot for $p27^{Kip1}$ (left panel) or $p21^{Cip1}$ (right panel). Graphs show averaged data obtained by densitometric analysis of four immunoblots. *p<0.05;**p<0.01 (one-sample t test)*

3.5 Cell cycle arrest in early S-phase

Evaluation of the results obtained so far indicated that resveratrol may arrest VSMCs in S- and not in G1-phase. The following experiments were performed to verify this hypothesis.

As demonstrated in Fig. 28, VSMCs require about 10-12 hours to reach S-phase and 22-24h to complete one cell cycle. Therefore, we assessed levels of

hyperphosphorylated Rb after 12 and 22 hours of calf serum stimulation in the presence of resveratrol. Aphidicolin, a well characterised inhibitor of DNA polymerase α and δ, was used as a positive control.

3.5.1 Comparison of resveratrol and aphidicolin regarding Rb hyper-phosphorylation and $p21^{Cip1}$ and $p27^{Kip1}$ expression

Neither resveratrol nor aphidicolin altered the Rb phosphorylation state until 12 hours, consistent with the idea that both compounds do not affect G1-phase. However, after 22 hours, both substances clearly increased hyperphosphorylated forms of Rb.

Similarly, the expression of $p21^{Cip1}$ and $p27^{Kip1}$ was not changed until 12 hours of treatment with aphidicolin or resveratrol, respectively. After 22 hours, $p27^{Kip1}$ levels appeared lower in response to both stimuli.

Interestingly, unlike resveratrol, aphidicolin did lower $p21^{Cip1}$ protein levels after 22 hours (Fig. 38). Of note, neither resveratrol nor aphidicolin altered basal levels of phosphorylated Rb, $p21^{Cip1}$ or $p27^{Kip1}$ (Fig. 38).

Taken together, these results suggest that resveratrol acts similarly to, but not identically with, the DNA polymerase inhibitor aphidicolin.

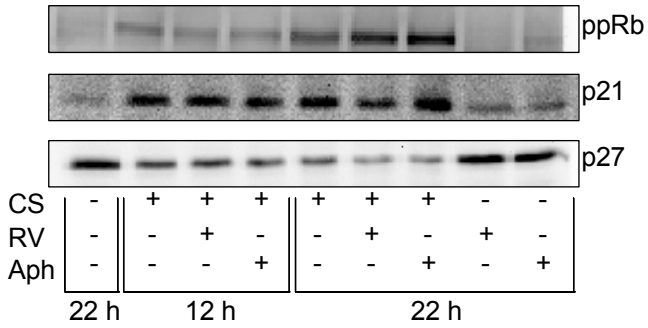

Fig. 38. Western blot analysis of Rb hyperphosphorylation, $p21^{Cip1}$ and $p27^{Kip1}$ protein expression. Quiescent VSMCs were treated with 100 µM resveratrol (RV), 1 µM aphidicolin (Aph) or vehicle for 30 minutes prior to stimulation with (+) or without (-) 10% calf serum (CS) for the indicated times. Panels show one representative Western blot out of three.

3.5.2 Resveratrol prevents cell cycle progression in early S-phase

To further corroborate the hypothesis that resveratrol causes a DNA replication block in S-phase, we determined whether resveratrol is still able to inhibit cell cycle progression when cells are already in S-phase. Therefore, we accumulated cells in early S-phase by employing aphidicolin. Subsequent treatment of released cells with resveratrol (or aphidicolin, as a positive control)

still prevented cells from synthesising DNA, as shown by the complete absence of BrdU incorporation (Fig. 39).

Thus, resveratrol is indeed able to arrest VSMCs in early S-phase.

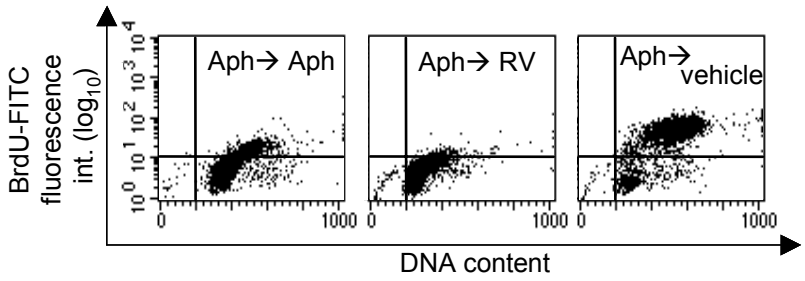

Fig. 39. Resveratrol inhibits S-phase progression of VSMCs at the G1/S-interphase. Quiescent VSMCs were synchronised in early S-phase by aphidicolin (Aph, 1 µM) treatment as described in Materials and Methods. After aphidicolin-release, cells were grown in fresh growth medium supplemented with 2 µM Aph, 100 µM resveratrol (RV) or vehicle for another four hours. During the last 30 minutes, cells were pulse labelled with BrdU and subsequently processed as described in Materials and Methods. Images show one representative experiment out of three.

3.6 Serine^{15}-phosphorylation of p53

Gottifredi *et al.* have recently shown that blocked DNA replication induces increased levels of serine^{15}-phosphorylated p53 that is, however, transcriptionally impaired as it is not accompanied by p21^{Cip1} accumulation (Gottifredi *et al.*, 2001). We, therefore, examined whether resveratrol leads to an increase in levels of serine^{15}-phosphorylated p53. Again, aphidicolin was used as a positive control.

Western blots depicted in Fig. 40 show a dramatic increase in serine^{15}-phosphorylated p53 after resveratrol as well as aphidicolin treatment. Consistent with the idea that resveratrol only transiently blocks DNA synthesis, the increase in phosphorylated p53 was not accompanied by an increase in p21^{Cip1} (Fig. 38) or Bax (Fig. 40) levels.

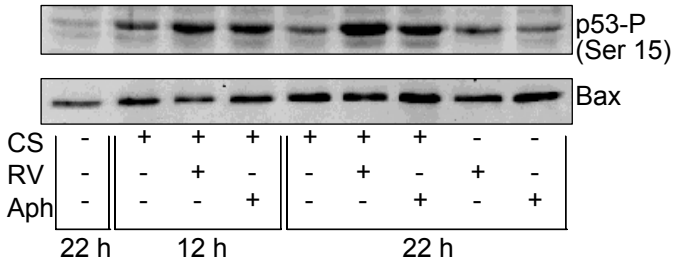

Fig. 40. Effects of resveratrol and aphidicolin and p53 serine[15] phosphorylation and Bax levels. Quiescent VSMCs were treated with 100 µM resveratrol (RV), 1 µM aphidicolin (Aph) or vehicle for 30 minutes before stimulation with (+) or without (-) 10% calf serum (CS) for the indicated times. Panels show one representative Western blot out of three.

3.7 γ-irradiation

Little is known about regulation of p53 in VSMCs. Therefore, to elucidate why enhanced p53 serine[15] phosphorylation by resveratrol is not accompanied by increased expression of target genes such as p21 or Bax in these cells, we performed additional experiments using γ-irradiation.

As expected, γ-irradiation alone as well as in combination with resveratrol or aphidicolin led to a strong increase in serine[15]-phosphorylated p53. However, in contrast to findings by Gottifredi et al. in RKO cells (Gottifredi *et al.*, 2001), neither resveratrol nor aphidicolin could prevent the augmentation of p21 levels after γ-irradiation. Interestingly, although resveratrol, aphidicolin and γ-irradiation increased p53 serine[15] phosphorylation in VSMCs, and γ-irradiation also p53 transactivation, total levels of p53 were not altered (Fig. 41).

These data suggest that γ-irradiation can rescue p53 from a transcriptional block induced by treatment of cells with aphidicolin or resveratrol.

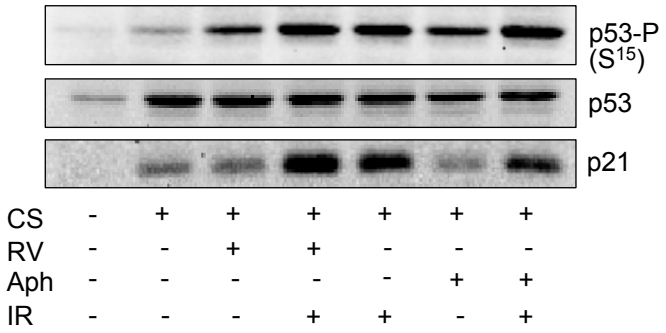

Fig. 41. Western blot analysis of serine[15] p53 levels, total p53 and p21 levels in resveratrol- or aphidicolin-treated and/or γ-irradiated VSMCs. Quiescent VSMCs were treated with growth medium containing 100 µM resveratrol (RV), 1 µM aphidicolin (Aph) or vehicle for 12 hours prior to γ-irradiation (IR, 10 Gy). Cells were harvested 12 hours after IR. Panels show one representative Western blot out of four.

E. Discussion

1 Influence of resveratrol on Ang II-treated VSMCs

Resveratrol is a polyphenolic stilbene derivate found in grape skin. It is proposed to be, at least in part, responsible for the beneficial cardiovascular effects attributed to red wine (Soleas *et al.*, 2001; Wu *et al.*, 2001). In this study, we show that resveratrol is able to attenuate VSMC hypertrophy induced by Ang II treatment, and provide insight into the molecular mechanisms that might be involved in mediating this effect.

1.1 Inhibition of Ang II-induced VSMC hypertrophy- involvement of kinases

Vascular hypertrophy is a critical determinant of vascular disease. Ang II is an important stimulus in this process, inducing protein synthesis but not DNA synthesis (Takahashi *et al.*, 1997; Schmidt-Ott *et al.*, 2000). Our results show that resveratrol attenuates [^{3}H]leucine incorporation in Ang II-stimulated cells and is therefore able to reduce VSMC hypertrophy.

The precise signalling mechanisms involved in mediating VSMC hypertrophy are not completely understood. However, it is known that phosphorylation and dephosphorylation of kinases plays an important role in regulating overall protein synthesis (Servant *et al.*, 1996). Ang II activates a plethora of protein kinases in VSMCs (Touyz and Schiffrin, 2000). Of these, the MAPKs Erk 1/2 and p38, the serine/threonine kinase Akt, as well as one of its downstream effector kinases, $p70^{S6k}$ (Eguchi *et al.*, 1999b), have been shown to mediate Ang II-induced hypertrophy (Ushio-Fukai *et al.*, 1998; Ushio-Fukai *et al.*, 1999; Servant *et al.*, 1996). Resveratrol attenuates phosphorylation of Erk 1/2, Akt and $p70^{S6k}$ but not p38 MAPK. In this regard, it has been shown that both the Erk 1/2 and the PI3-k/Akt pathway contribute to $p70^{S6k}$ activation in VSMCs (Eguchi *et al.*, 1999b). Interestingly, our results show that inhibition of the downstream kinase $p70^{S6k}$ by resveratrol is less potent than that of Akt. This is probably caused by signalling from the Erk 1/2 pathway to the $p70^{S6k}$, because resveratrol reduced Erk 1/2 phosphorylation far less effectively as it did Akt phosphorylation. Presumably, inhibition of both Akt and, to a lesser extent, the Erk 1/2 pathway, contribute to reduction of hypertrophy by resveratrol, as others have shown that inhibition of either pathway only partially inhibits hypertrophy (Servant *et al.*, 1996).

Regarding the mechanisms by which resveratrol may influence phosphorylation of Akt, two possibilities must be taken into account: either inhibition of

phosphorylation or activation of specific phosphatases. Akt is dephosphorylated and inactivated by the serine/threonine phosphatase 2A (Andjelkovic *et al.*, 1996; Millward *et al.*, 1999). However, pre-treating the cells with okadaic acid, an inhibitor of this phosphatase (Millward *et al.*, 1999), did not abrogate the inhibitory effect of resveratrol on Akt phosphorylation. Therefore, resveratrol does not seem to activate phosphatase 2A.

This leaves the possibility that resveratrol acts *via* the PI3-k pathway, since PI3-k activity is essential for Akt activation in Ang II-stimulated VSMCs (Eguchi *et al.*, 1999b). The PI3-k family can be divided into three classes, but for signal transduction upon growth factor stimulation, class IA PI3-k are most important (Vanhaesebroeck *et al.*, 2001). Class IA PI3-k are heterodimeric proteins composed of an 85 kDa regulatory subunit that is tightly associated with a 110 kDa catalytic subunit (Wymann and Pirola, 1998). Our results show that resveratrol is able to substantially reduce tyrosine phosphorylation of the PI3-k p85 subunit, an event previously correlated with PI3-k activity (Saward and Zahradka, 1997).

The kinase responsible for tyrosine phosphorylation of p85 is not yet identified, but autophosphorylation by PI3-k itself is unlikely since PI3-k has been reported to autophosphorylate on serine residues (Wymann and Pirola, 1998). Therefore, we conclude that resveratrol must act further upstream of PI3-k. This hypothesis is corroborated by the finding that, in contrast to the PI3-k inhibitor wortmannin, resveratrol was not able to abrogate Akt phosphorylation induced by calf serum treatment, implying some specificity for Ang- and EGF-stimulated pathways.

1.2 EGF-R transactivation

Transactivation of the EGF-R has been shown to be essential for early Ang II signalling through the AT_1-R (Kalmes *et al.*, 2001). The EGF-R is a receptor tyrosine kinase whose kinase domain is flanked by a carboxy-terminal tail that functions as an autoinhibitory substrate. Carboxy-terminal autophosphorylation is a prerequisite for EGF-R kinase activity. In the same time, these autophosphorylation sites are important for initiation of *downstream* signalling, serving as docking sites for various signalling molecules and complexes (Wells, 1999).

Consistent with results reported by others (Ushio-Fukai *et al.*, 2001a), we show a rapid increase in EGF-R phosphorylation upon Ang II stimulation. Several signalling pathways including Ca^{2+}, proline-rich tyrosine kinase 2 (PYK 2), metalloproteinases as well as ROS and c-Src kinase have been demonstrated to be involved in EGF-R transactivation (Eguchi *et al.*, 1999a; Eguchi *et al.*, 1998; Ushio-Fukai *et al.*, 2001a; Bokemeyer *et al.*, 2000; Kalmes *et al.*, 2001). However, resveratrol does not interfere with Ang II-induced EGF-R phosphorylation, indicating that resveratrol may act downstream of EGF-R.

This hypothesis is substantiated by the fact that Akt phosphorylation is still inhibited when EGF is used for stimulation.

1.3 Signalling downstream of EGF-R

Studies performed in other cell systems suggest that c-Src kinase is important for signalling downstream of EGF-R (Stover *et al.*, 1995; Sato *et al.*, 1995). Consistently, the c-Src kinase inhibitor PP1 completely blocked EGF-induced Akt phosphorylation. Interestingly, in HeLa cells, resveratrol has been shown to act as an inhibitor of c-Src (Yu *et al.*, 2001). Although c-Src is phosphorylated at various sites, phosphorylation of tyrosine^{418} in the kinase domain and tyrosine^{529} in the carboxy-terminus are most important in regulating its activity (see also Fig. 7). Phosphorylation of tyrosine^{529} stabilises intramolecular interactions between the carboxy-terminus and the SH2 domain, thereby repressing kinase activity. In contrast, autophosphorylation of tyrosine^{418} that is situated in the activation loop leads to increased kinase activity (Abram and Courtneidge, 2000). Phosphorylation on tyrosine^{215} in the SH2 domain has also been implicated in c-Src activation, impeding intramolecular binding of the carboxy-terminal inhibitory phospho-tyrosine^{529} and allowing thus kinase activation independent of carboxy-terminal dephosphorylation (Stover *et al.*, 1996).

We found that resveratrol, although capable of attenuating phosphorylation of tyrosine^{215}, has no impact on phosphorylation of tyrosine^{418}. Tyrosine^{215} phosphorylation, however, is dispensable for kinase activation. Tyrosine^{418}, on the other hand, is subject to intermolecular autophosphorylation and a positive regulator of c-Src kinase activity (Bjorge *et al.*, 2000). For that reason it seems likely that c-Src kinase in resveratrol treated cells remains active.

This hypothesis was further confirmed by the result that EGF-induced phosphorylation of tyrosine^{845} in the kinase domain of the EGF-R is not impaired when cells are pre-treated with resveratrol. Tyrosine^{845} is a highly conserved residue that is phosphorylated in a c-Src dependent manner and has been associated with increased EGF-R kinase activity (Tice *et al.*, 1999; Biscardi *et al.*, 1999; Ushio-Fukai *et al.*, 2001b). AG 1478 completely abolished phosphorylation on this site, consistent with the idea that c-Src has no access to this site unless the carboxy-tail is removed upon receptor autophosphorylation (Stover *et al.*, 1995). These observations, together with the fact that resveratrol is not able to reduce EGF-R transactivation, strongly indicate that resveratrol does not inhibit c-Src kinase activity.

We, therefore, postulate that resveratrol may interfere with a tyrosine kinase or phosphatase, as phosphorylation of tyrosine^{215} of c-Src and tyrosine phosphorylation of the p85 regulatory subunit are inhibited by this substance. It may also be speculated that resveratrol, although unable to inhibit overall

EGF-R tyrosine phosphorylation induced by Ang II, prevents phosphorylation of a single residue of the EGF-R or an adapter protein such as Grb 2-associated binder 1 (Gab 1) that is necessary for PI3-k signalling. In this regard, Zhang *et al.* have recently shown that induction of the tyrosine phosphatase Shp 2 negatively regulates EGF-dependent PI3-k activation by selective dephosphorylation of p85 binding sites on Gab 1, while PI3-kinase activation by other growth factors is unaffected (Zhang *et al.*, 2002). Further experiments will be performed in this lab to clarify a possible role of Shp 2 and Gab 1 in resveratrol signalling.

2 Influence of resveratrol on calf serum-treated VSMCs

Proliferation of VSMCs is critically involved in the development of vascular proliferative diseases (Braun-Dullaeus *et al.*, 1998; Dzau *et al.*, 2002). Resveratrol has been previously demonstrated to inhibit proliferation in various cell types (Soleas *et al.*, 2001). This study provides insight into the molecular mechanisms used by resveratrol to mediate its antimitogenic effects in VSMCs. We demonstrate that, while G1-phase is not affected, resveratrol leads to a reversible arrest in the S-phase of the cell division cycle.

2.1 Effects on Erk 1/2, $p70^{S6k}$ and Akt kinase phosphorylation

Cyclin D is the first cyclin to be induced when cells progress through G1-phase. D-type cyclins function as a link between mitogenic stimuli and the potentially autonomous cell cycle machinery. In this regard, the Erk 1/2 signalling cascade as well as Akt governed pathways contribute to the accumulation of cyclin D and its assembly with cdk4 and cdk6 (Sherr and Roberts, 1999). Thus, both pathways play a critical role in linking mitogenic cues to the G1-phase of the cell cycle. $p70^{S6k}$, situated downstream of Akt and Erk 1/2 (Eguchi *et al.*, 1999b), is thought to be implicated in cell cycle progression due to its capacity to phosphorylate the ribosomal protein S6 in response to mitogens. Although cell cycle analysis performed by flow cytometry and previously published data (Zou *et al.*, 1999) suggest that resveratrol might target G1-phase, we detected only slight inhibition of Akt and $p70^{S6k}$ phosphorylation. Erk 1/2 phosphorylation was not significantly altered at all, suggesting that factors other than these kinases mediate the antiproliferative effect of resveratrol. These results seem to contrast with the findings described in 1.1 showing that resveratrol inhibits phosphorylation of Akt, $p70^{S6k}$ and Erk 1/2, and with a study reporting inhibition of Erk 1/2 phosphorylation in endothelin-1-stimulated porcine coronary arteries pre-treated with resveratrol (El Mowafy and White, 1999). This apparent contradiction can be resolved considering the different proximal pathways induced by distinct stimuli. Besides that, it can be deduced that resveratrol targets neither of these kinases directly.

2.2 Effect on Rb hyperphosphorylation

Basically all signals relevant for G1 progression into S-phase finally culminate in Rb hyperphosphorylation. Rb senses and integrates a multitude of proliferative and antiproliferative signals by interacting with members of the E2F family of transcription factors (Weinberg, 1995; Trimarchi and Lees, 2002). In its hypophosphorylated, active state Rb forms a complex with E2F thereby blocking its transcriptional activation, and actively represses transcription of cell cycle genes (DePinho, 1998; Trimarchi and Lees, 2002). Hyperphosphorylation of Rb that is accomplished first by cyclin D-dependent kinases in mid-G1-phase and then completed by cyclin E-cdk2 leads to its dissociation from promoter-bound E2F, thus allowing transcription of E2F regulated genes (Sherr, 1996). Antimitogenic substances affecting G1-phase consequently prevent Rb hyperphosphorylation (Vinals *et al.*, 1999). In contrast to recent findings that resveratrol decreases the hyperphosphorylated form of Rb in human epidermoid carcinoma (A431) cells (Adhami *et al.*, 2001), we found that in serum-activated VSMCs, resveratrol strongly augments Rb hyperphosphorylation.

2.3 Effect on Cip/Kip proteins

So far, all results pointed more to the possibility of a resveratrol-mediated putative DNA replication block in early S-phase instead of an interference in G1. To investigate whether resveratrol indeed arrests cells in early S-phase, we determined levels of Rb hyperphosphorylation caused by resveratrol and, as a positive control, by the DNA polymerase inhibitor aphidicolin, after 12 and 22 hours of calf serum stimulation. As expected, neither resveratrol nor aphidicolin increases levels of Rb hyperphosphorylation until 12 hours. At this time point, cells are still at the G1/S-interphase and thus not affected by a putative S-phase block. After 22 hours, however, accumulation of resveratrol- or aphidicolin-treated cells in S-phase leads to a concomitant accumulation of hyperphosphorylated Rb as compared to normally progressing control cells. Consistently, also $p21^{Cip1}$ and $p27^{Kip1}$ levels seem to be altered only after prolonged treatment, corroborating that G1 is not affected by resveratrol and also not, as already known, by aphidicolin. Interestingly, in contrast to resveratrol, aphidicolin treatment does not downregulate $p21^{Cip1}$ protein levels, arguing against an identical mechanism of these two substances.

2.4 Inhibition of ribonucleotide reductase?

Cell-free *in vitro* studies have provided evidence that resveratrol is an inhibitor of DNA polymerase α and δ (Stivala *et al.*, 2001) and of ribonucleotide reductase (Fontecave *et al.*, 1998). Additionally, experiments performed in a fibroblast cell line revealed that the ribonucleotide reductase inhibitor

hydroxyurea also leads to accumulation of cells in early S-phase that is accompanied by an increase in hyperphosphorylated Rb (Linke *et al.*, 1996). So far, two distinct studies point to similarities between resveratrol and hydroxyurea in terms of ribonucleotide reductase inhibition (Fontecave *et al.*, 1998; Rodrigue *et al.*, 2001). Our results show, however, that resveratrol completely prevents further S-phase progression in cells released from aphidicolin block, suggesting that a preceding depletion of the deoxyribonucleotide pools is not a prerequisite for resveratrol-induced cell cycle arrest in VSMCs.

Taken together, resveratrol seems to block DNA replication in VSMCs similar to the DNA polymerase inhibitor aphidicolin.

2.5 Influence on p53 phosphorylation, expression and transactivation

The tumour suppressor protein p53 is critically involved in the cell cycle control and seems to play a key role in VSMC proliferation after vascular injury. In this context, loss of p53 activity has been implicated in the pathogenesis of human restenosis (Speir *et al.*, 1994), and transfer of wild-type p53 gene has been shown to inhibit VSMC proliferation *in vivo* and *in vitro* (Yonemitsu *et al.*, 1998). In normally proliferating cells, p53 is maintained at low levels. This is a consequence of its interaction with the Mdm2 protein, which targets p53 for ubiquitination and degradation. Stress signals such as γ-irradiation trigger p53 phosphorylation at a number of N-terminal sites, thereby affecting its affinity for Mdm2 and leading to p53 stabilisation (Oren, 1999). Phosphorylation at serine^{15}, in particular, has been implicated in this process (Oren, 1999; Shieh *et al.*, 1997).

However, more recent data suggest that serine^{15} phosphorylation does not alter p53 interaction with Mdm2. Instead, it is proposed to be responsible for p53 transactivation by promoting interaction with CREB binding protein (CBP)/p300 (Dumaz and Meek, 1999; Lambert *et al.*, 1998; Schon *et al.*, 2002). This seems to be true for VSMCs, since γ-irradiation, resveratrol and aphidicolin all cause enhanced serine^{15} phosphorylation, and γ-irradiation also transactivation, but fail to increase total levels of p53.

Another possible explanation for the missing upregulation of total p53 levels may lie in the early time point chosen for investigation; one study performed in VSMCs treated with x-rays shows an increase in total p53 levels after 4 days of treatment, and also Mayberg *et al.* report a delayed p53 induction after γ-irradiation of VSMCs (Mayberg *et al.*, 2000).

Our results show that a reversible DNA replication block triggered by substances such as aphidicolin or resveratrol leads to an increase in serine^{15} phosphorylation of p53. However, in striking contrast to DNA damaging events such as γ-irradiation, DNA replication block does not lead to enhanced levels of p21, a p53 regulated protein. This suggests that serine^{15}-phosphorylated p53

induced by agents blocking DNA replication is transcriptionally impaired, a finding that is consistent with results recently reported by Gottifredi *et al*. (Gottifredi *et al.*, 2001). Altogether, p53 serine^{15} phosphorylation is likely a consequence rather than a cause of growth inhibition, and serine^{15} phosphorylated but transcriptionally impaired p53 may thus be considered a marker for reversible S-phase arrest.

However, there seem to be considerable mechanistic differences between p53 regulation by aphidicolin in different cell lines: In RKO cells used by Gottifredi *et al.*, hydroxyurea and aphidicolin were able to suppress induction of p21 by γ-irradiation, suggesting that p53 is held in a state of active repression. This does not seem to be the case in VSMCs since γ-irradiation increases p21 levels despite the presence of aphidicolin or resveratrol, respectively. Therefore, it may be speculated that the molecular mechanism of action of resveratrol and aphidicolin is different in VSMCs compared to RKO cells: for VSMCs, active repression of p53 by aphidicolin and resveratrol appears less likely; instead, it seems probable that the impaired response to p53 after resveratrol and aphidicolin treatment may be due to the lack of critical modifications or cofactors of the p53 protein. This way, p53 can be considered as partially latent, with the full transcriptional activity being restored after γ-irradiation, a stimulus known to entirely activate the protein.

In summary, resveratrol and aphidicolin show very similar patterns regarding p53 modification alone as well as in combination with γ-irradiation. Hence, these results support the hypothesis that resveratrol induces a reversible S-phase arrest, with features very similar to the DNA polymerase inhibitor aphidicolin.

The impact of vascular proliferative diseases such as restenosis on morbitity and mortality in the developed countries explains the need to find new drugs that may help to reduce or prevent cardiovascular complications (Garas *et al.*, 2001; Dzau *et al.*, 2002). In this regard, insights into the mechanisms of action of a specific substance are indispensable for evaluation of its therapeutic potential. Resveratrol, based on data obtained in *in vitro* studies and animal experiments, has been suggested to be a potentially promising candidate (Wu *et al.*, 2001). This work provides insight into the signalling pathways that are modulated by resveratrol in VSMCs and may thus allow a better estimation of its therapeutic usability, although *in vivo* studies are certainly needed to permit a final appraisal.

F. Summary

The present work was intended to elucidate the molecular targets of resveratrol in Ang II- and calf serum-stimulated primary rat aortic VSMCs.

1 Effect of resveratrol on Ang II-stimulated VSMCs

Ang II is critically involved not only in modulating vasomotor tone but also in controlling functional and structural properties of the arterial wall.

Our results show that resveratrol reduces VSMC hypertrophy induced by Ang II, and that this effect is most likely caused by an interference with Akt- and, although to a lesser extent, Erk 1/2-governed pathways. Additional experiments revealed that resveratrol-mediated attenuation of Akt phosphorylation is not due to activation of the respective Akt-phosphatase but is rather based on inhibition of pathways *upstream* of Akt activation.

In this regard, our results provide evidence that c-Src kinase is not inhibited by resveratrol in these cells. Importantly, we were able to demonstrate that EGF-R transactivation, a very early event in Ang II signalling, is not affected by resveratrol. Consistently, Akt phosphorylation induced by EGF itself is also inhibited by resveratrol. On the other hand, Ang II-induced PI3-k tyrosine phosphorylation is substantially reduced by resveratrol pre-treatment, suggesting the molecular target of resveratrol to be located *downstream* of EGF-R but *upstream* of PI3k.

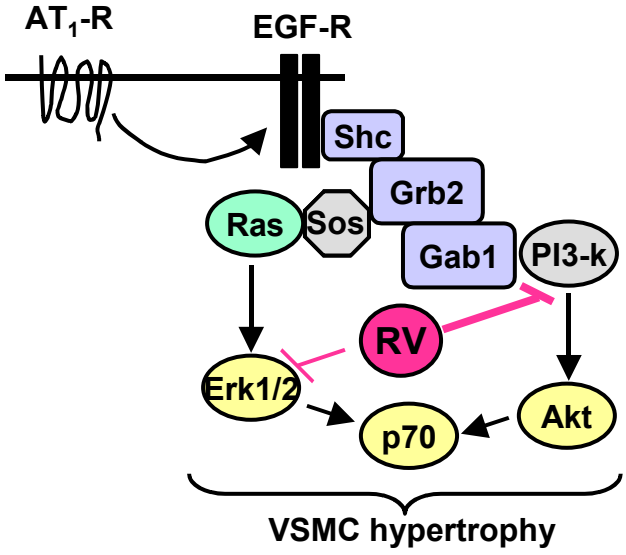

Fig. 42. Interference of resveratrol with pathways leading to Ang II-induced hypertrophy in VSMCs. Since the exact target of resveratrol is not yet identified, inhibition of the most upstream target in the signalling cascade is depicted.

2 Effect of resveratrol on calf serum-stimulated VSMCs

VSMC proliferation is considered a pivotal step in the genesis of cardiovascular diseases such as atherosclerosis and restenosis after angioplasty. Antiproliferative effects have been previously described for resveratrol, but seem to vary considerably depending on the cell type investigated.

We show that resveratrol reversibly inhibits cell cycle progression in early S-phase in calf serum-treated VSMCs. This effect is accompanied by an increase in hyperphosphorylated Rb while levels of the cdk-inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ are reduced compared to non treated control cells (see Fig. 43).

Furthermore, we were able to demonstrate that resveratrol-treatment of VSMCs leads to an increase in $serine^{15}$-phosphorylated p53 protein that is, however, transcriptionally impaired, since levels of p21 and Bax, two p53-regulated proteins, do not augment. Transcriptional impairment is likely caused by the lack of cofactors or critical modifications of the p53 protein, as additional application of γ-irradiation fully restores its transcriptional activity.

In summary, our results provide new insight into the molecular pathways influenced by resveratrol in VSMCs, and may help to better evaluate the potential of this substance as a possible therapeutic agent in cardiovascular disease.

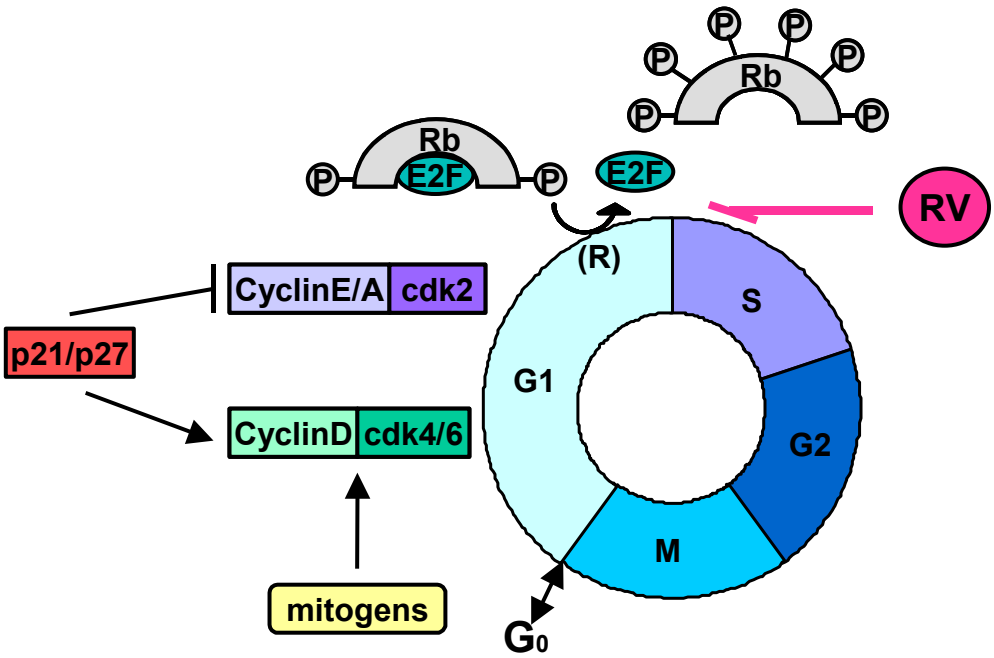

Fig. 43. Inhibition of cell cycle progression by resveratrol in calf serum-stimulated VSMCs. While signalling in G1-phase seems not to be affected, resveratrol (RV) treatment leads to a reversible cell cycle arrest in early S-phase.

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
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
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
Grb 2	growth factor receptor bound protein 2
Gab 1	Grb 2-associated binder 1
GSK 3	glycogen synthase kinase 3
Gy	Gray
h	hour
HB	heparin-binding
IL 6	interleukin 6
IP_3	inositol trisphosphate
IR	γ-irradiation
JNK	c-Jun N-terminal kinase
LDH	lactate dehydrogenase
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-diphenyl-tetrazolium 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

O.A.	ocadaic acid
p	phospho
PAA	polyacrylamide
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
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
PP2A	protein phosphatase 2A
PtdIns	phosphatidylinsositol
PTEN	phosphatase and tensin homologue deleted from chromosome 10
oxLDL	oxidised LDL
PDGF	platelet-derived growth factor
PYK 2	proline rich tyrosine kinase 2
R	restriction point
Rb	Retinoblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RV	resveratrol
S	serine
SDS	sodium dodecyl sulfate
SH1/2	Src homology 1/2
Shc	Src-homology 2 domain containing
SHIP	Src-homology 2-containing phosphatase
Sos	son of sevenless
T	threonine

TEMED	N,N,N´,N´-tetramethylethylenediamine
TGF-β	transforming growth factor β
TOP	tract of oligopyrimidines
TRIS	Tris(hydroxymethyl)-aminomethane
v	volume
VSMC	vascular smooth muscle cell
w	weight
WM	wortmannin
Y	tyrosine
µ	micro

2 Alphabetical order of companies

Acros Organics	Schwerte, Germany
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
Peske	Aindling-Pichl, Germany
Roche	Mannheim, Germany

Roth	Karlsruhe, Germany
Serotec	Eching, Germany
Sigma	Taufkirchen, Germany
Tocris	Ellisville, USA
Upstate Biotechnology	Lake Placid, USA

3 Publications

3.1 Abstracts

Haider UGB, Sorescu D, Griendling KK, Vollmar AM, Dirsch VM

Resveratrol inhibits proliferation and cell cycle progression of rat aortic smooth muscle cells – involvement of Akt kinase?

Free Radical Biology and Medicine, Vol 31, Suppl 1, 2001.

Haider UGB, Sorescu D, Griendling KK, Vollmar AM, Dirsch VM

Resveratrol reduces angiotensin II-induced hypertrophy in rat vascular smooth muscle cells in part by interfering with PKB/Akt and Erk 1/2.

Archieves of Pharmacology, Vol 365, Suppl. 1, 2002. Lecture

3.2 Original publications

Haider UGB, Sorescu D, Griendling KK, Vollmar AM, Dirsch VM

Resveratrol Suppresses Angiotensin II- Induced Akt/Protein Kinase B and p70 S6 Kinase Phosphorylation and Subsequent Hypertrophy in Rat Aortic Smooth Muscle Cells.

Mol Pharmacol. 2002 Oct;62(4):772-7

Haider UGB, Sorescu D, Griendling KK, Vollmar AM, Dirsch VM

Resveratrol increases serine^{15}-phosphorylated but transcriptionally impaired p53 and induces a reversible DNA replication block in serum-activated vascular smooth muscle cells.

Mol Pharmacol, in press

Haider UGB, Sorescu D, Griendling KK, Vollmar AM, Dirsch VM

Resveratrol inhibits angiotensin II-mediated Akt activation but does not impede EGF-receptor transactivation.

Manuscript in preparation

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