Mechanistic studies on resveratrol and its influence on angiotensin II- and epidermal growth factor-induced signaling pathways in vascular smooth muscle cells
Gewidmet allen, die mich unterstützt haben
Abstract

In the developed societies cardiovascular disease (CVD) is the leading cause of death. Angiotensin II (Ang II) contributes to the development of CVD by acting as a potent vasoconstrictor, but also as growth factor and by promoting endothelial dysfunction. In vascular smooth muscle cells (VSMC), Ang II promotes cell growth (hypertrophy). It was recently shown that the red wine polyphenol resveratrol (RV) inhibits Ang II-induced hypertrophy by interfering with the PI3K/Akt pathway. This was suggested to occur by the activation of the phosphatase SH2-domain containing phosphatase 2 (Shp2), a redox-sensitive molecule. The current work is based on the hypothesis that RV can restore Shp2 activity by interfering with reactive oxygen species (ROS) produced downstream of the activated epidermal growth factor receptor (EGF-R) that is transactivated in VSMC in response to Ang II. Inhibition of ROS production will prevent oxidation and thus inhibition of Shp2. Specific aims of this thesis were to examine a) whether RV acts as antioxidant when inhibiting Akt phosphorylation in Ang II- and EGF-activated VSMC, b) to identify the source of ROS responsible for Akt activation downstream of the EGF-R, c) to verify the role of Shp2 as a RV target in VSMC and to determine whether RV affects Shp2 by a redox-sensitive mechanism.

In this study we indeed show an antioxidative effect of RV, as it inhibits intracellular ROS in Ang II-activated VSMC and also basal ROS levels. When testing the dependence of Ang II- and EGF-induced phosphorylation/activation of major signalling molecules such as Akt, p38 and ERK1/2 on ROS, we found that Ang II-induced phosphorylation of Akt and p38 is dependent on ROS produced by Nox1, whereas EGF-stimulated phosphorylation of these kinases was shown to occur redox-independent. Using non-redox active derivates of RV in this setting underlined that antioxidative properties of RV are not necessary for the inhibition of phosphorylation of Akt. Approaching the role of Shp2 in the molecular mechanism of RV in VSMC we were unable to detect changes in the oxidation status of Shp2 but revealed differences in phosphorylation status of Shp2 and PI3K recruitment to Shp2 upon Ang II or EGF treatment, suggesting a distinct regulation of Shp2 upon Ang II or EGF stimulation. This study indicates that the antioxidant activity of RV is not required for the strong Akt inhibitory/antihypertrophic activity of RV in Ang II-activated VSMC.
Zusammenfassung

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B  INTRODUCTION
B Introduction

1. Atherosclerosis

1.1. Pathogenesis of atherosclerosis

Responsible for about 50% of all deaths in the developed societies, cardiovascular disease (CVD) is the leading mortal disease worldwide. Atherosclerosis commonly results from the combination of an unhealthy environment, genetic susceptibility and increased lifespan in our society. In the beginning of atherosclerosis there is chronic injury to endothelial cells (EC) through shear stress, hypertension or other stimuli. Due to increased endothelial permeability low-density lipoproteins (LDL) as well as platelets and blood monocytes cross the vessel wall and accumulate in the intima. LDL are then modified via oxidation to produce oxLDL. This initiates the development of early lesions, consisting of subendothelial accumulations of cholesterol-enriched macrophages (“foam cells”) generated by uptake of oxLDL (Fig. 1A). Then the lesion is advancing to “fatty streaks” by enrichment of lipid-rich necrotic debris and VSMC, which are recruited from the media into the intima by growth factors or proliferate in the intima directly, secreting large amounts of extracellular-matrix components. By recruiting more monocytes and leukocytes to the atherosclerotic lesions a chronic inflammation is perpetuated via secretion of chemokines, cytokines and reactive oxygen species (ROS). This again favours the uptake of oxidized LDL in macrophages, release of pro-inflammatory cytokines and recruitment of inflammatory cells (Fig. 1B). The

Figure 1: Initiation and progression of atherosclerosis
The first step in the development of atherosclerosis is the formation of a fatty streak (A), which can further lead to an intermediate lesion (B); then development of a lesion that is vulnerable to rupture can follow (C) and in the end an advanced obstructive lesion can narrow the vessel (D). Figure from Rader D.J. et al.8
originating plaque contains a central lipid core consisting of cholesterol, separated from the arterial lumen by a fibrous cap which is produced by VSMC (Fig. 1C). Upon rupture of the plaque thrombogenic material is exposed in the lumen leading to a thrombus or by continued growth of the plaque the lumen is narrowed.1-4 (Fig.1D)

1.2. Role of vascular smooth muscle cells (VSMC)

Situated in the media of a healthy blood vessel the VSMC usually remain in a quiescent state being mainly responsible for vasoconstriction and dilation. As a consequence of injury, which also leads to increased exposure to cytokines and growth factors, VSMC change their phenotype into a proliferative one, migrate into the intima and form a so called neointimal layer. By excretion of pro-inflammatory mediators and vascular cell adhesion molecules they contribute to inflammation. Synthesis of matrix molecules by VSMC leads to a retention of lipoproteins and VSMC also produce a firm fibrous cap which promotes plaque formation and consequently intimal thickening.2,5-7 Proliferating and hypertrophic VSMC play a dominant role in the development of atherosclerosis. When cultivated they also change their phenotype to a proliferative and ECM disposing one which makes subcultured VSMC a suitable model for cells found in the neointima8.

2. Resveratrol

2.1. Source and history

The polyphenol resveratrol (3,5,4’-trihydroxy-trans-stilbene; RV) (Fig. 2) is a phytoalexin which is synthesized in spermatophytes in response to injury, UV irradiation and fungal attack.9 It has been found in about 70 plants like grapes, mulberries and peanuts, and was first isolated from roots of white hellebore (Veratrum grandiflorum O.Loes) in 1940. Positive effects of RV on health were initially claimed in 1963, when it was identified as the active constituent of Polygonum cuspidatum (Ko-jo-kon), a traditional Asian medicine10. RV became famous after it was discovered in red wine, postulating that it can explain some of the cardioprotective effects of red wine11. The phenomenon, known as the “French Paradox”, describes the inverse correlation between red wine consumption and incidence of
cardiovascular disease shown in epidemiological studies. In France the incidence of heart infarction is about 40 % lower than in the rest of Europe, although the diet is traditionally rich in saturated fat.\textsuperscript{12}.

### 2.2. Health promoting effects of resveratrol

#### 2.2.1. Vasoprotection

RV can prevent platelet aggregation \textit{in vitro}\textsuperscript{13} and was also reported to block increasing platelet aggregation \textit{in vivo}\textsuperscript{14}, further it can reduce atherosclerotic areas and the size of generated thrombi\textsuperscript{15} by modulating COX1 over COX2 activity\textsuperscript{16}. A vasorelaxant activity of RV was found in isolated arteries and rat aortic rings\textsuperscript{17, 18}, mediated by multiple pathways such as stimulating Ca\textsuperscript{2+}- activated K\textsuperscript{+} channels and enhancing endothelial nitric oxide synthase (eNOS) as well as inducible nitric oxide synthase (iNOS)\textsuperscript{19}. Also low-density lipoprotein (LDL) particles can be protected from oxidation by RV as it can chelate copper or scavenges free radicals\textsuperscript{20, 21}.

#### 2.2.2. Antioxidant activity

It was shown that phenolic compounds in red wine such as RV have antioxidative activity and can therefore prevent LDL oxidation\textsuperscript{22}. Intracellular and extracellular ROS can be significantly inhibited at a concentration of 1-100 \( \mu \text{M} \) RV. Reason for this effect is that RV can scavenge free radicals\textsuperscript{23, 24} and in VSMC it was also shown to enhance endogenous antioxidants such as catalase\textsuperscript{15, 25} and to elevate glutathione, a cellular antioxidant, by influencing its viability and oxidation\textsuperscript{23}. In addition, RV is able to increase endogenous antioxidants and phase 2 enzymes in cardiomyocytes which leads to an enhanced cellular...
defence mechanism against oxidative injuries. The hydroxyl groups of RV seem to be important for the antioxidant activity and the 4’ position was determined as the most important one for this effect, whereas the 3- and 5- OH groups were shown to act in a synergistic way. Also the trans-isomery and the double bond in the stilbenic skeleton play a crucial role for the antioxidant effects of RV in cells. Recent studies revealed that RV derivatives with additional hydroxyl groups like piceatannol (PCA; 3,5,3’,4’-tetrahydroxy-trans-stilbene) that is produced by a cytochrome P450 catalyzed hydroxylation of RV show an enhanced anti-radical activity. In contrast deletion of the hydroxyl group at the 4’position of RV reduced its antioxidant activity.

2.2.3. Cancer

Augmented research on RV and cancer began after the first report of a beneficial outcome when treating skin tumours topically with RV. Several publications described the inhibitory potential of RV on initiation and growth of tumours in a wide variety of rodent cancer models. RV can impair cancer development in various ways: by inhibiting carcinogen activation, induction of carcinogen detoxifying enzymes, modulating cell survival and apoptosis, inhibition of angiogenesis and blocking metastasis. Currently, Phase I clinical trials are underway including one study where RV is given to patients with colon cancer and another for safety and the pharmacokinetic profile of repeated administration of RV.

2.2.4. Aging and sirtuins

Sirtuins or Sir (silent information regulator) 2-like proteins belong to a conserved family of NAD+-dependent histone (class III histone)/protein deacetylases (HDAC). They can deacetylate chromatin and therefore create transcriptionally silent chromatin. Sirt 1-7 were also found to be able to deacetylate non-histone proteins such as p53, Forkhead box-O transcription factors (FOXO) and α-tubulin at lysine residues. Deacetylation by HDAC of these residues are often a prerequisite for subsequent ubiquitination and protein degradation. Lately RV was found to extend the lifespan of S. cerevisiae, Caenorhabditis elegans and Drosophila melanogaster, and also of a species of short-lived fish. The proteins which are thought to be responsible for this phenomenon are the yeast Sir2. Also its mammalian homolog SIRT1 is linked to extending life, as knock out mice lacking SIRT1 had a reduced lifespan. Furthermore, RV was found in an initial in vitro screen as the most
potent of 18 inducers of SIRT1 deacetylase activity\textsuperscript{35}. These effects of RV on SIRT1 were not reproducible when not using a specifically modified substrate \textit{in vitro}\textsuperscript{39}, though RV was able to upregulate the Sirt1 enzyme \textit{in vivo}.\textsuperscript{40}

2.2.5. Resveratrol and Angiotensin II

Ang II plays an important role in controlling cardiovascular homeostasis and development of cardiovascular diseases\textsuperscript{41}. Previous studies of our groups showed already a regulatory effect of RV on Ang II-treated cells. In VSMC RV was able to inhibit proliferation and interfere with Ang II-induced hypertrophy.\textsuperscript{42-44} Publications using cardiomyocytes showed that Ang II-mediated hypertrophy was inhibited by ROS attenuation of RV\textsuperscript{45}. Furthermore, RV was able to interfere with \textit{in vivo} cardiac hypertrophy by modulation of NO and ET-1 production.\textsuperscript{46} In cardiac fibroblasts an Akt-independent inhibition of proliferation and differentiation after Ang II treatment was detected by RV\textsuperscript{47}. A recent publication showed that RV attenuated Ang II-induced interleukin (IL)-6 expression via suppression of the IL-6 gene promoter activity.\textsuperscript{49}

2.2.6. Bioavailability

Due to a rapid metabolism, RV has a short initial half-life of about 8 to 14 min\textsuperscript{50,51}. \textit{In vivo} studies showed a very low intestinal uptake of RV and therefore minor concentrations in the blood due to extensive metabolism in the gut and liver\textsuperscript{52}. When given intravenously, RV is converted to sulphate conjugates within \textit{~}30 min in humans\textsuperscript{53}. Although the concentrations of \textit{trans}-RV in red wine vary widely, a daily intake of 375 ml wine of a person weighing 70 kg would correspond to a RV consumption of \textit{~}27 \textmu g/kg per day when calculated with a RV content of 5 mg/l, leading to a plasma level concentration of up to 100 nM. A higher dose of red wine, however, could interfere with the beneficial effects of RV as negative effects of ethanol increase.\textsuperscript{9}

We used a rather high dose of RV (50 \textmu M) in our experiments, as the aim of this work was not to mimic physiological effects of RV but to investigate the molecular action of RV in VSMC.
3. Angiotensin II

3.1. Physiological action

Angiotensin II (Ang II) is a potent growth factor in VSMC, known to induce hypertrophy but not hyperplasia in cultured VSMC. As the main player in the renin-angiotensin system (RAS), the vasoactive peptide angiotensin II (Ang II) is a potent enhancer of blood pressure via inducing vasoconstriction. As a critical hormone it further affects the function of all organs, including heart, kidney and brain. Clinical studies and pharmacological investigations about the effect of angiotensin-converting enzyme (ACE) inhibitors revealed that Ang II plays a central role in pathophysiology, inducing among others hypertension, cardiac hypertrophy, vascular thickening, and atherosclerosis. Ang II is triggering various actions in VSMC. Besides modulating vasomotor tone it regulates cell growth and apoptosis, influences cell migration and extracellular matrix deposition, acts proinflammatory and stimulates production of other growth factors (e.g. Platelet-derived growth factor PDGF) and vasoconstrictors (ET-1). In rats chronic Ang II infusion leads to a hypertension and hypertrophy.

3.2. Ang II receptors

The G protein-coupled Angiotensin type 1-4 receptors (AT1-4R) have been identified as target receptors of Ang II. In VSMC the seven transmembrane regions containing-AT1-R is known to be responsible for most physiological effects. This receptor is widely distributed in all organs, like liver, adrenals, brain, kidney, lung, heart and vasculature. Ang II-induced hypertrophy in VSMC are mediated via the AT1-receptor (AT1-R), which can transactivate the epidermal growth factor (EGF)-, PDGF- and insulin-like growth factor (IGF) receptors. In rodents two subtypes named AT1A-R and AT1B-R are present, sharing 94 % homology, whereas in humans only the AT1-R is found. Short-time increase of Ang II leads to an elevated level of AT1-R activation. However, after chronic exposure to Ang II these receptors are downregulated. When stimulated, the AT1-R is endocytosed within 10 minutes after activation. Approximately 25 % of the internalized receptors are recycled back to the plasma membrane, the remainder is degraded in the lysosomes.
3.3. Cellular signal transduction

After binding of Ang II to the AT1-R the signal can be transduced by activation of receptor tyrosine kinases (EGF-R, PDGF-R, IGF-R) and non-receptor tyrosine kinases (c-Src family kinases, Ca\textsuperscript{2+}-dependent proline-rich tyrosine kinase 2 (Pyk2), focal adhesion kinase (FAK) and Janus kinases (JAK)).\cite{60} Findings also show that the activation of NAD(P)H oxidases and generation of reactive oxygen species (ROS) can be induced by Ang II stimulation\cite{69} leading to activation of several serine/threonine kinases such as PKC, MAPK kinases (ERK1/2, p38 and c-jun N-terminal kinase (JNK))\cite{60}, whereas other studies did not show a clear dependence of MAPK on ROS production\cite{58,70,71}.

3.3.1. AT1-R related signaling

The AT1-R consists of seven transmembrane domains and interacts with multiple heterodimeric G-proteins (G\textsubscript{q/11}, G\textsubscript{i}, G\textsubscript{12}, G\textsubscript{13}) producing second messengers including ROS.\cite{72} The vasoconstrictor effect of Ang II is mediated by G-protein-coupled activation of downstream effectors like phospholipase C (PLC), phospholipase A2 (PLA2), and phospholipase D (PLD). Following PLC activation inositol-1,4,5-triphosphosphate (IP3) and diacylglycerol (DAG) are produced within seconds, leading to calcium efflux into the cytoplasm via IP3 binding and subsequently opening of Ca\textsuperscript{2+}-channels on the sarcoplasmic reticulum. By recruiting Ca\textsuperscript{2+} to myosin light chain kinase (MLCK) the interaction between actin and myosin is increased, the VSMC contract.\cite{60,65,72} One main signaling pathway of Ang II is the MAP kinase pathway, including extracellular signal-regulated kinase (ERK1/2), JNK and p38, which cause VSMC differentiation, proliferation and migration.\cite{60,73} ERK1/2 is quickly activated by Ang II treatment via calcium-dependent kinase Pyk2 phosphorylation of Src and its adaptor protein Shc further leading to the formation of a Shc/Growth factor receptor binding protein (Grb2) complex. This in turn leads to recruitment of SOS (son of sevenless) and Raf, a scaffold protein which associates with the small GTP-binding protein Ras. Once activated Ras favours phosphorylation and subsequently activation of the MAPK/ERK kinase (MEK).\cite{41,58,60} In addition to MAPK activation Ang II stimulation also influences the apoptosis signal regulating kinase 1 (ASK1), which subsequently induces JNK and p38 related signaling\cite{74}. Activation of the MAPK cascade influences cell survival, apoptosis and differentiation.\cite{75}
4. EGF and EGF-R

The family of epidermal growth factors (EGF) consists of several peptide growth factors, among which EGF and heparin-binding EGF (HB-EGF) are known to bind to the EGF-receptor (EGF-R). Before becoming active and binding to receptors, all members of this family have to be cleaved and only after processing by a disintegrin and metalloproteinase (ADAM) proteins the inactive precursor, a transmembrane protein, becomes active. HB-EGF was found to be a chemoattractant produced by VSMC and macrophages and was also shown to influence migration and proliferation of VSMC. EGF is mainly secreted by bone marrow platelets, but capable of binding to the EGF-R of VSMC and EC. Effects of EGF

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**Figure 3: Simplified overview of the Ang II signaling in VSMC**

Ang II regulates cell survival, growth and hypertrophy by activation of Noxes and subsequently transactivation of the EGF-R or activation of the MAPK pathway downstream of the AT1R directly. The EGF-R itself can activate the MAPK as well as the PI3K/Akt signaling pathway. Adopted from Metha P.K. et al and Higuchi S. et al.

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**Introduction**
 appeared to be more effective in intimal VSMC than in medial SMC. In the vasculature EGF and HB-EGF are important in atherogenesis as they mediate the transformation of VSMC from a differentiated non-proliferative into a dedifferentiated proliferative and migratory phenotype, supporting the development of atherosclerosis.

The EGF-R is a prototype of the type-1 growth factor receptor family with tyrosine kinase activity. Four subtypes of EGF-R are known including ErbB1 (EGF-R, HER1), ErbB2 (HER2, p185), ErbB3 (HER3, p160) and ErbB4 (HER4) and all of them are expressed in VSMC. The EGF-R can either be activated by binding EGF or HB-EGF, the production of the later is induced by Ang II stimulation. Upon ligand binding to the extracellular domain of the receptor a conformational change is induced leading to dimerization of the EGF-R monomers. Subsequently the five important intrinsic protein tyrosines become autophosphorylated, activating the intrinsic EGF-R tyrosine kinase domain. Also transphosphorylation of the EGF-R by Src was shown. The phosphorylated tyrosine residues on the receptor can be bound by Src homology 2 (SH2) domain-containing proteins, initiating the MAP kinase- and also PI3K-signaling pathway. Known binding partners of the EGF-R at distinct tyrosine residues at the C-terminus are: phospholipase C gamma (PLCγ), phospholipase D (PLD), Src, growth factor receptor-bound protein 2 (GRB2), GRB2-associated binder-1 (Gab-1), SH2 containing phosphatase-1 (Shp-1) and Ras. The activation of the EGF-R is leading to various signaling cascades, e.g. ERK and p38 activation has been shown to depend on EGF-R transactivation, whereas the JNK-pathway is EGF-R independent. Another pathway, whose activation through the EGF-R is well established is the PI3K dependent phosphorylation of Akt.

For the strength of the signaling not only ligand binding to the receptor but also the abundance of the receptor on the cell surface seems to be important. This can be influenced by the cell by receptor shuttling, internalization and degradation. At the membrane the EGF-R is found integrated into caveolae (cholesterol-rich membrane regions containing high amounts of the protein caveolin), although data in literature are contradictory. Ligand binding then leads to a EGF-R shuttling to lipid rafts (cholesterol-rich membrane regions), which are important for signaling as here several other signaling molecules are found co-localized.
4.1. Transactivation of the EGF-R by Ang II

The EGF-R can not only be activated by stimulation with EGF directly. Also Ang II can rapidly transactivate the EGF-R\textsuperscript{88}. In addition it was shown that also H\textsubscript{2}O\textsubscript{2}\textsuperscript{89}, endothelin\textsuperscript{90}, thrombin\textsuperscript{91}, oxidized LDL\textsuperscript{92} and mechanical stretch\textsuperscript{93} can lead to an activation of the EGF-R in cultured VSMC\textsuperscript{94}. The EGF-R transactivation by Ang II requires ROS\textsuperscript{95} and upstream kinases such as c-Src or cAbl\textsuperscript{72}, occurring in cholesterol-rich domains of caveolae\textsuperscript{96}. Also evidence for a dependence on calcium were found\textsuperscript{60}. The activation of c-Src and cAbl lead to release of HB-EGF by metalloproteinases (ADAMs)\textsuperscript{72}. Recently ADAM17 was identified in VMSC to be responsible for the transactivation\textsuperscript{97}. The EGF-R is phosphorylated mainly at Tyr\textsuperscript{1173} and Tyr\textsuperscript{1068} by Ang II-induced ROS production\textsuperscript{70,95}. Phosphorylated Tyr\textsuperscript{1068} is the binding site for the Grb2/Shc/SOS complex. This favours the activation of Ras/Raf/ERK1/2 as well as the induction of the PI3K/phosphoinositide dependent kinase-1 (PDK1)/Akt cascade, inducing cellular metabolism, growth, survival and remodelling\textsuperscript{60,72,98}.

5. Insulin

Insulin is another peptide inducing VSMC proliferation and growth via the PI3K/Akt and MAPK pathway. It is a hormone synthesized and secreted in pancreatic β- cells in response to an increased level of glucose in the blood. By activating insulin receptors (IR) insulin controls glucose, lipid and protein metabolism. Similar to its homologous peptide insulin-like growth factor (IGF-1) insulin also exhibits functions in the cardiovascular tissue such as increased vasorelaxation by stimulating NO release and reduced Ca\textsuperscript{2+} dependent contraction. Unlike insulin, IGF-1 can be produced by cardiomyocytes and VSMC themselves in response to Ang II and other growth factors or mechanical forces\textsuperscript{99}. Following the receptor activation members of IR substrate (IRS) family, consisting of IRS-1 to IRS-4, are phosphorylated\textsuperscript{100}. Two major signaling pathways are then induced by insulin: the MAPK pathway and the PI3K/Akt pathway. The recruited IRS serves as docking protein for SH2 domain-containing molecules such as PI3K, which interacts via its p85 regulatory subunit with the IRS, resulting in an increase of the catalytic activity of the p110 subunit. Also growth factor receptor binding protein 2 (Grb2) and Shp2 can bind the IRS, triggering the SOS induced activation of the Raf/MAPK cascade\textsuperscript{101}. The PI3K pathway is responsible for most metabolic actions of insulin, whereas the MAPK pathway regulates gene expression and cell growth\textsuperscript{102}. A connection of the Insulin signaling and Ang II signaling was discovered as Ang II inhibiting agents in insulin resistant patients did not only reduce blood pressure but
also improved insulin sensitivity\textsuperscript{102}. In VSMC several reports show that Ang II can impair the insulin-mediated IRS-1 tyrosine phosphorylation and IRS-1/PI3K association\textsuperscript{103}. Also an influence of Ang II on the insulin signaling by proteasome-dependent degradation of IRS-1 by Src, PDK1 and ROS-mediated phosphorylation of IRS was shown\textsuperscript{104,99}.

Figure 4: Insulin signaling pathway
A schematic overview of the signaling induced by Insulin binding to the insulin receptor (IR), which is leading to autophosphorylation of the receptor and subsequently recruitment of IRS and other molecules such as Shc. Activated downstream pathways contribute to lipid synthesis, glycogen synthesis, protein synthesis and glucose uptake. Adopted from Olivares-Reyes J.A. et al\textsuperscript{102}. 
6. PI3K

The phosphatidylinositol 3’ kinase (PI3K) is a key player for transducing signals downstream of the EGF-R, leading to an activation of Akt and mTor and subsequently hypertrophy. As a kinase, it is able to phosphorylate the 3’-OH position of the inositol ring of phosphoinositides. When activated the members of the Class I PI3K are phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP2) to generate Phosphatidylinositol-3,4,5-trisphosphate (PIP3), which are both bound to the membranes. Proteins, which possess the PH (pleckstrin homology) domain, are then recruited to the plasma or endosomal membrane by PIP3. Here they either transactivate other proteins or activate themselves. The PI3K consists of a regulatory adapter subunit (p85) coupled to a catalytic subunit (p110α, β, γ). PI3K activity is negatively regulated by two phosphoinositide phosphatases. One is the Src homology 2 (SH2) domain-containing inositide phosphatase (SHIP), the second is phosphatase and tensin homolog deleted on chromosome ten (PTEN), both converting PIP3 to PIP2. In the EGF-R signaling PI3K is recruited to tyrosine phosphorylated adaptor protein Gab1, which is bound to Tyr1068 and Tyr1086 at the EGF-R. Gab1 is located at the plasma membrane via its PH domain and therefore PI3K is also recruited to the membrane by Gab1-binding, producing PIP3 out of PIP2. This is leading to more Gab1 recruitment to the plasma membrane as it can bind to PIP3 via its PH domain, favouring Gab1-PI3K recruitment leading to an increase of the signal via a positive feedback loop.

7. Akt

In response to growth factor and hormone stimulation the serine/threonine kinase Akt/PKB is a critical signaling node in all higher eukaryotic cells. Three isoforms are known - Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). They are highly homologous to protein kinases A, G and C, sharing the structure of their catalytic domain. Akt1 is ubiquitously expressed, Akt2 is found predominantly in insulin target tissues such as fat cells, liver and skeletal muscle and Akt3 is rarely expressed, mainly in the brain. Important properties of Akt are the PH domain, that allows the molecule to be recruited to the membrane, and the hydrophobic motif (HM) domain, acting as docking site for kinases such as PKC and p70S6K. Upon activation of receptor tyrosine kinases (RTK), scaffolding adaptors bind to the receptor, recruiting and activating PI3K that produces PIP3. Both, Akt and PDK1 can bind to PIP3 at the plasma membrane and PDK activates Akt by phosphorylation at Tyr308. In addition, also the so called kinase PDK2 is recruited which phosphorylates Akt.
at Ser\textsuperscript{473}. It is not absolutely clear at the moment, which kinase PDK2 really is but evidence is increasing that the mTOR complex 2 (mTORC2) is acting as PDK2.\textsuperscript{110-113} The first known substrate of Akt was glycogen synthase kinase 3 (GSK3), which is keeping cell cycle-related targets as cyclin D, c-Myc and the eukaryotic initiation factor (eIF) 2B in an inactive status. Upon phosphorylation by Akt GSK3 is inactivated leading to cell cycle progression and protein synthesis\textsuperscript{114}. The GSK3 was also found recently to modulate apoptosis favouring survival by inhibition of the Bcl-2 family member MCL-1 and interfering with caspase-9 activation\textsuperscript{115} as well as BAD activation\textsuperscript{116}. Akt mediates phosphorylation and export of members of the FOXO-family of forkhead transcription factors out of the nucleus causing their inactivation and therefore blocking target genes that promote apoptosis, cell-cycle arrest and metabolic processes\textsuperscript{106, 117;109}. Moreover, Akt induces cell growth by mTORC1 dependent activation of S6 kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1).\textsuperscript{109} Protein synthesis is regulated by Akt via mTOR dependent p70S6 kinase activation, inducing transcription factors AP-1 and E2F.\textsuperscript{105}

8. MAPK

The mitogen-activated protein kinases (MAPK) are a ubiquitous and highly conserved family of serine-threonine protein kinases activated by environmental stimuli including growth factors, cytokines, shear stress, vasoactive agents, and oxidative stress.\textsuperscript{118-120} Extracellular-signal regulated kinases (ERKs), p38MAPK (p38\textsubscript{α}, p38\textsubscript{β}, p38\textsubscript{γ}, p38\textsubscript{δ}) and c-Jun N-terminal kinases (JNKs) are the three major members of this family and important for an injury caused vascular response. MAPK are regulated by phosphorylation; organised in a triple kinase pathway in which MAPK serve as substrates for MAPK kinases (MKK), which in turn are phosphorylated by MAPK kinase kinases (MKKK). When the EGF-R is activated, Grb2 and the constitutively associated SOS are recruited to the plasma membrane, where SOS catalyses the switch of membrane-bound inactive Ras to GTP-bound active Ras. p120-RasGAP is a GTPase-activating protein containing SH2 domains that counteracts SOS-dependent activation of Ras.\textsuperscript{121} Ras activates Raf1 which then phosphorylates ERK1/2 on a threonine and tyrosine residue.\textsuperscript{119, 122} Substrates of the activated ERK1/2 are found at the plasma membrane, in the cytosol and in the nucleus, leading for example to the induction of transcription factors Elk1, c-Myc and the p90 ribosomal S6 kinase\textsuperscript{123}. The GTP-bound Ras can in addition activate JNK and p38 cascades, stress induced stimuli such as ROS were shown to activate JNK and p38 independent of Ras\textsuperscript{122}. Importance for p38 and ERK1/2 for aortic injury was indicated by several studies where balloon injury of rat carotid artery lead to a rapid activation of ERK1/2, p38 \textalpha/\textbeta MAPK and JNK 1/2, promoting neointima.
formation and VMSC proliferation. In the development of atherosclerosis JNK2 and p38 α activity is regulating the expression or activity of proteins required for internalization of oxLDL prior to their binding to cell-surface receptors.

9. **Protein tyrosine phosphatases (PTP) in the VSMC signaling**

In cells the response to external signals is often mediated by reversible phosphorylation of intracellular proteins. Protein tyrosine phosphatases (PTP) such as PTEN, DEP1, and Shp1 gain more and more importance in cancer as tumour suppressor genes. The PTP Shp2 was identified so far as the phosphatase that acts as a proto-oncogene. Phosphatases are also related to many other diseases including autoimmunity and developmental disorders. Phosphatases are able to counteract the phosphorylation by removing the phosphates which were transferred to a protein by kinases before. Due to their specificity for target amino acids the phosphatases can be divided into two major groups, the Ser/Thr phosphatases and the Tyr phosphatases, some phosphatases also possess dual specificity. The PTP share the active-site signature motif HCX₅R, including a cystein residue which is a nucleophile and essential for catalysis. Due to their microenvironment the catalytic cysteins (C-S⁻) have a low pKa, and under normal conditions they form thiolate anions, highly susceptible to oxidation. The transfer of the phosphate moiety from the substrate to the catalytic cysteine and rapid hydrolysis of the phosphate is important for the function of PTPs. When the cystein is oxidised its nucleophilic property is abrogated inhibiting of PTP activity. Many stimuli are already found to inactivate PTP transiently by producing ROS. The reversible oxidation leads to formation of sulphenic acid (C-SOH), whereas oxidation to sulphinic (C-SO₂H) or sulphonic (C-SO₃H) acids are usually irreversible. To prevent cysteins from oxidation PTP can form a disulfide bridge containing the catalytic cystein or sulphenic-amide bonds as it is the case for PTP1B or Shp2. Transactivation of a receptor, like the Ang II induced activation of the EGF-R is often combined with H₂O₂ generation, resulting in PTP redox inactivation and consequently PTK redox activation. A redox dependent activation of the PDGFR by Ang II transactivation suggests to be dependent on redox activation of Src and Pyk2 kinases and also PTP redox inhibition. This tight regulation is achieved by a localized ROS production specifically inactivating a single phosphatase and varying among cells.
9.1. Src homology 2 (SH2) domain containing phosphatase 2 (Shp2)

One PTP important in the Ang II signaling in VSMC is the 68 kDa big cytosolic phosphatase Shp2. Together with its parologue Shp1 it shares a specific structure unique in the PTP family, namely two SH2 domains positioned at the N-terminus of the molecule. This structure is followed by the catalytic domain and a C-terminal tail important for regulatory functions which contains tyrosine and serine phosphorylation sites. In addition Shp2 has a proline-rich domain that might bind SH3 domain-containing proteins like Src and Lyn, but lacks the nuclear localization sequence (NLS) Shp1 possesses. Shp1 is mainly known as negative regulator of signaling important for haematopoietic mechanisms, while Shp2 is regarded as a positively acting key factor in development since inactivation of...
Shp2 in knock out experiments led to major developmental defects.\cite{121, 141} For the neointima development a regulatory effect of Shp2 is suggested as it was found to be upregulated during neointima formation in VMSC.\cite{142}

9.1.1. Posttranslational modification of Shp2 - oxidation and phosphorylation

The main ways of regulation of Shp2 are phosphorylation at the residues Tyr\textsuperscript{542}, Tyr\textsuperscript{580}, Ser\textsuperscript{576} and Ser\textsuperscript{591}, oxidation of the cystein residue at the catalytic site\cite{140} or binding proteins via SH2 domains. Under basal conditions Shp2 is catalytically inactive due to SH2 domain-mediated autoinhibition, as its N-terminal SH2 domain is inserted into the catalytic cleft\cite{139}. By binding of the SH2 domains to a tyrosine phosphorylated ligand the PTP activity increases dramatically.\cite{130} The two tyrosine residues (Tyr\textsuperscript{542}, Tyr\textsuperscript{580}) are then susceptible to

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\textbf{Figure 6: Shp2 structure and regulation}

\textit{A, Schematic structure of Shp2.} Shp2 contains two SH2 domains, a N-terminal and a C-terminal SH2 domain, a catalytic PTP domain and a C-terminal tail containing two tyrosine residues. \textit{B, Potential mechanism of Shp2 regulation.} In basal state Shp2 is largely inactive due to a “backside loop” of the N-SH2 inserted in the catalytic cleft. Phosphorylation at the two SH2 domains by either a SH2-binding protein (BP) or by binding the two phosphorylated residues Tyr\textsuperscript{542} and Tyr\textsuperscript{580} at the C-terminus leads to activation of Shp2 which results in induced binding and dephosphorylation of substrates (S). Adopted from Neel B.G. et al.\cite{143} and Shen K.\cite{139}
phosphorylation by different receptors and non-receptor protein tyrosine kinases (PTK) and other proteins including insulin response substrate-1 (IRS-1), and Gab1. Two major functions of Shp2 have been proposed including acting as adaptor to recruit proteins such as Grb2 and SHIP through their SH2 domains, as well as direct signal regulation via its phosphatase activity. PKC activation leads to a serine phosphorylation of Shp1 and Shp2, but Shp2 activity seems not to be affected by this action. The tyrosine phosphorylation of Shp2 seems to be dependent on the stimulus, FGF and PDGF induce it, in contrast EGF seems not to do so, indicating a role for the phosphorylation in some but not all signaling pathways. Currently, it is unclear which of the tyrosine residues of Shp2 are important for the activity, as contradictory data is found in the literature. As described above, Shp2 is prone to oxidation at the catalytic cystein residue, resulting in an inactive catalytic domain whereas redox regulation of Shp2 seems do be very much dependent on the cell type. A recent publication indicates a redox-dependent regulation of Shp2 by Ang II via oxidation and phosphorylation in VSMC.

9.1.2. Shp2 and EGF-R signaling

Knock out experiments in mice suggest a pivotal role for Shp2 in the EGF-R signaling in vivo as Shp2 chimeric knock out cells show a phenotype similar to the EGF receptor knockout mice. In EGF induced signaling docking sites for the phosphotyrosine-binding domains (SH2), which also Shp2 possesses, is created by autophosphorylation of the EGF-R. In the EGF signaling it was shown that the EGF-R is dephosphorylated by Shp2 on Tyr922, a RasGAP-binding site, abolishing the binding of this Ras inhibitor to the EGF-R and thus, excluding it from the EGF-induced signaling complex and promoting MAPK activation. Later it was shown that this effect is mediated by dephosphorylation of the binding site for RasGAP on Gab1 by Shp2. In addition to the well known activation of MAPK after EGF stimulation Shp2 plays a role in the PI3K/Akt signaling pathway regulation. By dephosphorylation of Gab1 at the binding site for the PI3K subunit p85 it can inhibit PI3K binding, leading to an inhibition of the Gab1-PI3K positive feedback loop, as shown by the interference of Shp2 with PI3K activity and Akt phosphorylation in fibroblasts. Activation of Shp2 by RV was shown in fibroblasts by our own group previously. A recent paper also connects Shp2 to Ang II signaling and the EGF-R by findings in COS cells showing that Shp2 activity is important for the direct interaction of the AT1-R and the EGF-R. Recently also an interaction of c-Src and Shp2 was detected in endothelial cells after Ang II stimulation, influencing Nox and ERK1/2 activity.
10. **ROS**

Reactive oxygen species (ROS) are products of the normal cellular metabolism. In former times ROS was regarded as toxic compound, triggering apoptosis and cell damage. ROS was also found to be produced during the respiratory burst in neutrophils for bacterial killing. Nowadays a more complex view of ROS function is accepted as lower concentrations of ROS are involved in cell signaling, leading to cell growth and differentiation, to modulation of the ECM, and to inactivation of NO and many kinases. The concentration of ROS is kept below a toxic threshold by fine balance of oxidants and antioxidants in the cell.\textsuperscript{157,158} Members of the ROS family normally contain unpaired electrons. The superoxide anion (O$_2^-$) is the so called “primary” ROS; produced in the vessels mainly by NADPH oxidases, the “secondary” ROS include hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^\cdot$) and nitric oxide (NO).\textsuperscript{159, 160} Other sources of ROS in VSMC are xanthine oxidase, lipoxygenase and nitric oxide synthase. H$_2$O$_2$ is cell-permeant, very stable and generated primarily by superoxide dismutase (SOD)-mediated dismutation of O$_2^-$\textsuperscript{161, 162} Intracellular regulators of ROS are catalase and glutathione peroxidase (Gpx1). Catalase is found mainly in peroxisomes, catalysing the reaction of H$_2$O$_2$ to water, Gpx1 is located in the cellular cytosol and mitochondria, favouring also the detoxification of H$_2$O$_2$ to water by producing glutathione disulfide out of glutathione.\textsuperscript{161}

10.1. **ROS in Ang II signaling**

ROS play an important role as mediators of Ang II signaling. Ang II-induced hypertrophy of VSMC is suggested to be NADPH oxidase-dependent\textsuperscript{163}. H$_2$O$_2$ derived from NADPH oxidases was shown to be needed for the activation of c-Src\textsuperscript{164} and also transactivation of the EGF-R seems to be redox-dependent. The detailed mechanism is not known but Nox1 seems to be important for ROS production after Ang II treatment. Several kinases are susceptible to ROS, a redox regulation of p38 and JNK has been shown clearly, whereas the data for ERK1/2 are more contradictory.\textsuperscript{161} H$_2$O$_2$ treatment was shown to induce the phosphorylation of Akt\textsuperscript{166} and this phosphorylation is further thought to be redox regulated as p38 can phosphorylate and recruit MAPKAPK-2 to the Akt/p38 complex, leading to a phosphorylation of Akt on Ser\textsuperscript{473, 167}.
11. NADPH oxidases

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, in short “Noxes”, are the most important producers of ROS in VSMC. The only ROS source in mammalian cells was thought for a long time to be the NADPH oxidase of phagocytes (Phox), found mainly in neutrophils and macrophages where it catalyses the respiratory burst. The function of the Nox there is to produce large amounts of superoxide upon exposure of the cell to microorganisms or stimulation by inflammatory mediators.\textsuperscript{162, 168, 169} In other cells Noxes were found to produce ROS in a rather regulated manner. There are at least three different Noxes present in the vasculature: Nox4 is expressed ubiquitously, Nox1 is highly expressed in VSMC and Nox2 is mainly expressed in endothelial cells and cardiomyocytes.\textsuperscript{170, 171} Table I shows the expression of different Noxes in tissues.\textsuperscript{168, 172, 173}

<table>
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<th>Activation</th>
<th>High level of expression</th>
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<tr>
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<td>Nox5</td>
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<tr>
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<td>lymphoid tissue, endothelium, VSMC</td>
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</tbody>
</table>

Table I: Nox expression in tissues

11.1. Structure and activation

The Nox2 protein (also called gp91phox) was the first Nox identified in phagocytes. Meanwhile seven other Nox family members were found, all of which are important for the ROS production.\textsuperscript{172} The Nox prototype is a membrane-integrated glycoprotein which further contains two hemes in the NADPH-binding site located at the very COOH terminus, and FAD-binding domains are located in the cytoplasmic region, making it capable of transporting electrons from NADPH via FAD to molecular oxygen.\textsuperscript{174} Homologues of gp91phox in human and animals are called Nox1-Nox5. In addition there were also dual oxidases (Duox) found, named Duox1 and Duox2, which are Ca\textsuperscript{2+} dependent and contain a peroxidase-homology domain. This enables Duox to both generate reactive oxygen and use it
through its own peroxidase domain\textsuperscript{169, 171, 175} In animals Nox1-Nox4 form a heterodimer with the membrane protein p22phox, a complex known in phagocytes as flavocytochrome b\textsubscript{558}. The activation of Nox1 and Nox2 by interaction with p22phox together with the cytosolic subunits leads to a production of superoxide by sequential transfer of two electrons from NADPH to molecular oxygen.\textsuperscript{165, 169}

\subsection*{11.2. Nox1}

Ang II was the first reagent shown to stimulate ROS production via Nox1 activation in rat VSMC using it in pathophysiologically relevant concentrations.\textsuperscript{162, 176} The main ROS species produced by this pathway is superoxide (\(O_2^{-}\)).\textsuperscript{177, 178} Activation of the membrane bound Nox1 protein is dependent on the interaction with p22phox, an other membrane-located protein, and recruitment of the cytosolic subunits p47phox and p67phox or their homologues to the Nox1 complex via their SH3 domain. In addition the interaction of the small GTPase Rac with the other Nox subunits is important for activation. p47phox is thought to organize the translocation of the other cytosolic factors to the membrane and therefore its homologues are designed as “organizer subunit” (NOXO1). After translocation to the membrane p67phox, the “activator subunit”, or its homologue NOXA1, are then activated by contact to Nox1 and this subsequently leads to Rac interaction with Nox1.\textsuperscript{172} In VSMC the involvement of p67phox or NOXA in Nox1 activation is not well defined in literature.\textsuperscript{172, 179}

The detailed mechanism of Nox activation by Ang II is not absolutely clear. It was shown that the activation of p47phox via phosphorylation by protein kinase C (PKC) and subsequently translocation of the membrane is needed.\textsuperscript{179} Upon stimulation with Ang II the AT1-R binds to caveolin-1 leading to trafficking of the AT1-R from high-density non-caveolar membrane fractions into caveolin-1-containing caveolae/lipid rafts where Nox1 can be found. Rac1 has to be activated and recruited to caveolae/lipid rafts for maintaining the Nox activity.\textsuperscript{176, 178, 180} Possibly Rac is activated by c-Src via activation of c-Abl and SOS-1 that in turn translocates Rac to the lipid rafts and activates it.\textsuperscript{181} A role for PI3K in Rac-1 activation is suggested since Ang II-triggered ROS release can be inhibited by PI3K inhibitors.\textsuperscript{164} Finally, this localized ROS production seems to be necessary for EGF-R transactivation.\textsuperscript{178}
Figure 7: Structure and activation of Nox1 in VSMC

For the activation of Nox1 in VSMC the cytosolic subunits p47phox (NOXO), Rac and possibly also NOXA (p67phox) have to be translocated to the two membrane-bound subunits Nox1 and p22phox. The activation results in the formation of superoxide (O$_2^•^-$), which is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide (H$_2$O$_2$). The intracellular antioxidant catalse can decompose H$_2$O$_2$ to oxygen and water. ROS regulates the expression and activation of several mediators of cardiovascular disease. Adopted from Guzik T.J. et al.180, Cai H. et al.162 and Garrido et al.179.

Also other stimuli were shown to activate Nox1. Proliferation of VSMC in response to growth factors like urokinase plasminogen activator (uPA) was shown to be dependent on Nox1.182, 183 Basic fibroblast growth factor (bFGF)184 and PDGF induced migration was shown to be is Nox1-related.185 In addition, in VSMC Nox1 was shown to contribute to p38 and Akt activation186, 165, 171 Nox1 knock out mice were shown to develop less Ang II-induced hypertension187, whereas conflicting observations were made concerning Nox1 involvement in hypertrophy.187, 188

Introduction
11.3. Nox4

For Nox4 activation only binding of p22phox to Nox4 is needed rendering Nox4 constitutively active. Rac binding to the complex seems not to be important but findings in the literature are controversial\textsuperscript{189,131}. Therefore Nox4 is responsible for the basal ROS level in VSMC, and it was shown that Nox4 is important for the maintenance of the differentiated phenotype of the VSMC.\textsuperscript{190} In contrast to other Noxes, the main ROS produced by Nox4 after Ang II treatment is hydrogen peroxide (H$_2$O$_2$).\textsuperscript{177}

Studies show that after vascular injury, during hypoxia and serum deprivation the Nox4 protein expression was strongly induced.\textsuperscript{171, 184} Other data indicated that stimuli like Ang II, thrombin and PDGF induce Nox1 expression but down regulate Nox4 expression level.\textsuperscript{168, 171,165} In contrast to Nox1 which is always membrane bound Nox4 was found recently in distinct subcellular locations in VSMC, mainly in the nucleus and at focal adhesions where also activated EGF-R, PI3K and Src can be found.\textsuperscript{95, 181, 191} When the EGF-R and caveolin-1 are phosphorylated due to a growth factor signal by c-Src they appear together with Nox4 and paxillin at focal adhesions, supposedly forming a redox signaling platform.\textsuperscript{178}
12. Aim of the work

In the western world cardiovascular disease (CVD) is the leading cause of death. One important factor contributing to the development of this disease is angiotensin II (Ang II). Ang II acts as a potent vasoconstrictor and growth factor and can promote endothelial dysfunction. In vascular smooth muscle cells (VSMC), Ang II promotes cell growth (hypertrophy). It was recently shown that resveratrol (RV) inhibits Ang II-induced hypertrophy in VSMC by interfering specifically with the PI3K/Akt pathway. The main site of action was reported to be downstream of the epidermal growth factor-receptor (EGF-R) that was activated either by EGF stimulation directly or via transactivation of the receptor by Ang II. In fibroblasts, evidence was found that RV increases the activity of the redox-sensitive SH2-domain containing phosphatase 2 (Shp2). As active Shp2 can dephosphorylate the PI3K binding site at Grb-2 associated binder (Gab) 1 and therefore at the EGF-R, PI3K recruitment to the receptor is impaired and Akt activation is hampered (Fig. 8). The current work is based on the hypothesis that RV can restore Shp2 activity by interfering with reactive oxygen species (ROS) produced downstream of the activated epidermal growth factor receptor (EGF-R) that is transactivated in VSMC in response to Ang II. Inhibition of ROS production will prevent oxidation and thus inhibition of Shp2. Specific aims of this thesis were to:

(i) examine whether RV acts as antioxidant when inhibiting Akt phosphorylation in Ang II- and EGF-activated VSMC.
(ii) identify the source of ROS potentially responsible for Akt activation downstream of the EGF-R.
(iii) examine whether Ang II and EGF-triggered phosphorylation of Akt occurs redox dependent.
(iv) verify the role of Shp2 as a RV target in VSMC and to determine whether RV affects Shp2 by a redox-sensitive mechanism.
Figure 8: Hypothesis
The EGF-R can be activated either by EGF stimulation directly or by Ang II-mediated transactivation. In fibroblasts it was shown that Shp2 can dephosphorylate the PI3K binding site at Gab1, leading to impaired PI3K recruitment to the receptor and subsequently inhibition of Akt activation. RV could influence Shp2 activity by reducing ROS produced by Noxes upon stimulation.
C MATERIALS AND METHODS
C Material and Methods

1. Chemicals and buffers

All chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) and Carl Roth (Karlsruhe, Germany).

2. Cell culture

2.1. Isolation

Vascular smooth muscle cells (VSMC) were isolated from three different aortas of male Sprague-Dawley rats. The aortas were excised from rats and placed in PBS −, then cleaned from connective tissue and incised longitudinally. After incubation in digestion buffer for 15 min at 37 °C the adventitia and the endothelium were scrapped off and the aortas were minced. The aortas were incubated in digestion buffer at 37 °C for 3 h to complete the enzymatic digestion. Afterwards the cells were pelleted by centrifugation (10 min, 1100 rpm, 25°C) and seeded with growth medium in a 25 cm² flask. The cells were verified as VSMC by fluorescence microscopy using a monoclonal anti-α-smooth muscle actin FITC-conjugated antibody (1:250 dilution, Sigma).

2.2. Cultivation

Cells were cultured at 37 °C and 5 % CO₂ in a phenol red-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % calf serum (CS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. VSMC were passaged twice a week, grown at about 90 % confluence. For harvesting cells were treated with trypsin/EDTA, reaction was stopped with 10 % CS containing DMEM followed by centrifugation for 4 min at 1,400 rpm. Then cells were seeded in 75 cm² flasks. For experiments cell passages 7 to 14 were used, plated
in 6-well plates, 6 cm, 10 cm or 20 cm dishes. For stimulation the cells reached a confluence of 60 - 80 %. Prior to stimulation cells were serum-starved with DMEM containing 0.1 % calf serum for 24 h.

2.3. Storage

For freezing VSMC were pelleted by centrifugation, resuspended in ice-cold freezing medium (containing 10 % DMSO). Approximately 1 x 10⁶ cells/ml were frozen in cryovials, first at -20 °C for one day, then -80 °C for three days before they were stored in liquid nitrogen at -196 °C. When thawing the cells the cryovial was warmed to 37 °C and immediately suspended in warm growth medium, pelleted by centrifugation to remove the trypsin and seeded in 75cm² flasks.

Prior to usage the calf serum (CS) was inactivated by heating to 56 °C for 30 min. Aliquots were stored at -20 °C.

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<th>Provider</th>
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<td>Calf serum</td>
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Table II: Cell culture reagents
## Solutions for cell culture

**Solutions: cell culture**

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<td>EDTA</td>
</tr>
<tr>
<td>Freeze medium</td>
<td>DMEM</td>
</tr>
<tr>
<td></td>
<td>Calf Serum (CS)</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
</tr>
<tr>
<td>Digestion buffer</td>
<td>Collagenase (246 U/mg)</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td></td>
<td>Gentamicin sulphate</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
</tr>
<tr>
<td></td>
<td>Ham’s F12 medium (PANTM biotech)</td>
</tr>
</tbody>
</table>

Table III: Cell culture solutions
2.4. **α-actin staining of VSMC**

For identification of VSMC the cells were stained with a FITC-conjugated anti-α-smooth muscle actin antibody. Cells were grown on cover slips for which they were transferred into 12-well plates and 2 x 10⁵ cells/well were seeded on them. After growing over night the cells were washed once with ice-cold PBS and then fixed with methanol/acetone (1:1) for 15 min at 4 °C. Cells were washed twice with PBS and incubated with the anti-α-smooth muscle actin antibody for 60 min at 4 °C. Before fixing the VSMC-containing cover slips on a microscopy slide the cells were washed again with PBS. Pictures were taken with an Olympus BX51 microscope, using the FITC filter at a 400-fold magnification.

2.5. **Solutions for treatment of VSMC**

VSMC were grown in 6 cm, 10 cm or 20 cm dishes until they reached about 60 – 80% confluence. Then they were stimulated with Ang II, EGF, Insulin or H₂O₂ for the indicated times. RV, inhibitors and blocking peptides were normally preincubated 30 min before stimulation if not stated otherwise. Control cells were treated with 0.1 % DMSO as vehicle control when RV was used.

2.5.1. **Ang II solution**

Ang II (Sigma) was dissolved to a 2 mM stock solution in 0.25% BSA in PBS, stored at -80 °C. For working aliquots, then stock solution was diluted in 0.25% BSA to a concentration of 20 μM and stored at -20 °C. All experiments were performed using 100 nM Ang II for 10 min, when not noted otherwise.

2.5.2. **EGF**

The EGF (Upstate) solution was prepared in PBS at a concentration of 100 μg/ml and stored at -20 °C. For experiments cells were stimulated with 100 ng/ml for 5 min unless stated differently.
2.5.3. **Insulin**

An Insulin solution from bovine pancreas (10 mg/ml, Sigma-Aldrich) was used and diluted prior to stimulation to a concentration of 100 nM in DMEM without supplements.

2.5.4. **Resveratrol and its derivatives**

A trans-Resveratrol (Sigma) stock solution with a concentration of 100 mM was prepared in DMSO and stored at -80 °C. For treatment of the cells RV in a final concentration of 50 μM was preincubated with the cells for 30 min.

The resveratrol derivatives Tri-O-methylresveratrol (TM-RV) and 3,5-Dihydroxy-4’-methylstilben (R1) were synthesized by the group of Prof. Erker (Department of Pharmaceutical Chemistry, University of Vienna) and dissolved as described above.

### Inhibitors and antioxidants

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG 1478 (Tyrphostin)</td>
<td>250 nM</td>
<td>Sigma</td>
</tr>
<tr>
<td>AEBSF</td>
<td>10 μM - 1 mM</td>
<td>Sigma</td>
</tr>
<tr>
<td>DMTU</td>
<td>50 μM - 10 mM</td>
<td>Sigma/Fluka</td>
</tr>
<tr>
<td>DPI</td>
<td>10 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>ds-tat control</td>
<td>100 μM</td>
<td>Sigma/Caslo</td>
</tr>
<tr>
<td>EUK 134</td>
<td>50-200 μM</td>
<td>Cayman</td>
</tr>
<tr>
<td>gp91 ds-tat</td>
<td>100 μM</td>
<td>Caslo</td>
</tr>
<tr>
<td>Indirubin-3’-monoxime</td>
<td>3 μM</td>
<td>Laurent Meijer (Roscoff)</td>
</tr>
<tr>
<td>MnIII TM</td>
<td>50-200 μM</td>
<td>Cayman</td>
</tr>
<tr>
<td>MPG</td>
<td>50-300 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>NAC</td>
<td>10 mM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sirtinol</td>
<td>10 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>50 nM</td>
<td>NEB</td>
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Table IV: Inhibitors and antioxidants

"Materials and Methods"
### Blocking peptides

<table>
<thead>
<tr>
<th>NoxI</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp91 ds-tat</td>
<td>[H]-RKKRRQRRRRCSTRIRRQL-NH2</td>
</tr>
<tr>
<td>ds-tat control</td>
<td>[H]-RKKRRQRRRAGAGAGAGA-NH2</td>
</tr>
</tbody>
</table>

### 3. Protein lysates

Cells were stimulated as described, then put on ice, washed twice with ice-cold PBS and freshly prepared lysis buffer (110 μl/6 cm dish) was applied on the cells immediately. Cells were lysed on ice at 4 °C for 30 min; proteins were scraped off and lysates were cleared of all cell components by centrifugation at 13,000 rpm for 10 min at 4 °C. After taking a small volume for protein quantification supernatant was diluted 1:3 with 3 x SDS sample buffer (containing 15 % 2-mercaptoethanol), boiled for 5 min at 95 °C and stored at -80 °C.

### Buffers for protein lysates

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer (stock solution)</td>
<td>HEPES</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>NaF</td>
</tr>
<tr>
<td></td>
<td>Na&lt;sub&gt;3&lt;/sub&gt;HP&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;7&lt;/sub&gt; x 10 H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
</tr>
<tr>
<td></td>
<td>Na&lt;sub&gt;3&lt;/sub&gt;VO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>ddH&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td>Prior to use</td>
<td>Stock solution</td>
</tr>
<tr>
<td></td>
<td>PMSF 0.1 M</td>
</tr>
<tr>
<td></td>
<td>Complete™ 25x</td>
</tr>
<tr>
<td></td>
<td>Trition X-100 (10%)</td>
</tr>
<tr>
<td>WB sample buffer stock</td>
<td>Tris-HCl (0.5 M; pH 6.8)</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
</tr>
<tr>
<td></td>
<td>Bromphenol blue</td>
</tr>
<tr>
<td>3x sample buffer</td>
<td>WB sample buffer stock</td>
</tr>
<tr>
<td></td>
<td>2-mercaptoethanol</td>
</tr>
</tbody>
</table>

Table VI: Buffers for protein lysates
3.1. Protein quantification

The amount of protein was determined using the method of Bradford. The protein lysates were diluted in ddH₂O 1:10. 10 μl were transferred into a 96-well plate, each sample was measured in triplicates. A standard curve with BSA (50-500 μg/ml) was prepared and 190 μl Bradford dilution was added to standards and samples. The plate was incubated for 5 min and the absorbance at 595 nm was measured using a Tecan Sunrise™ microplate reader.

3.2. Immunoprecipitation

Cells were seeded in 10 cm dishes and stimulated as described. Proteins were solubilized with 500 μl lysis buffer/10 cm dish and cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Homogenates were incubated with the primary antibody (1 μg anti-Shp2) over night at 4 °C followed by 3 h incubation with 30 μl Protein A/G PLUS-Agarose beads (Santa Cruz), which were diluted 1:1 in PBS. Beads were spun down and washed three times with lysis buffer before adding 20 μl sample buffer (15 % 2-mercaptoethanol).

4. SDS-PAGE and Western Blotting

4.1. SDS-PAGE

To separate the protein samples a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli was performed. Therefore polyacrylamide (PAA) gels were polymerized using a 30 % solution of PAA/0.8 % bisacrylamide and a Mini-PROTEAN™ 3 Cell System (BIO-RAD). Final concentration of all used gels was depended on the molecular weight of the protein of interest. Mainly 10 % gels were used. The gel was loaded with equal amounts (20 μg) of proteins which were separated by electrophoresis at 30 mA/gel for up to 2 h.
4.2. Western blotting and detection

The separated proteins were transferred to a PVDF membrane using the tank blotting technique by applying 100 V for 90 min in a Mini Trans-Blot™ Electrophoretic Transfer Cell System (BIO-RAD). Membranes were then blocked with 5 % fat-free milk powder.
in TBS-T for at least 1 h and washed three times with TBS-T for 10 min. Specific primary antibodies were added to the membranes and incubated at 4 °C overnight. Before adding horseradish-peroxidase conjugated secondary antibodies the membranes were washed three times with TBS-T for 10 minutes. The secondary antibodies were incubated for at least 1 h at room temperature. The membranes were washed again three times with TBS-T before visualization of the protein bands using ECL-solution to detect proteins with a LAS-3000™ (Fujifilm) luminescent image analyzer. The band intensity was determined by densitrometric analysis using AIDA software (raytest).

**Antibodies and beads**

<table>
<thead>
<tr>
<th>Target</th>
<th>Source</th>
<th>Dilution</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>phospho Akt S473</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>Erk1/2</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>phospho Erk1/2 Y202/204</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>Oxidized PTP Active Site</td>
<td>mouse, mc</td>
<td>1:500</td>
<td>R&amp;D Systems (Minneapolis, MN, USA)</td>
</tr>
<tr>
<td>p38</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>p85 (PI3K)</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>Upstate (Charlottesville, VA, USA)</td>
</tr>
<tr>
<td>phospho p38 Y180/182</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>SH-PTP2 (C-18)</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td>phospho-SHP-2 Y542</td>
<td>rabbit, pc</td>
<td>1:1000</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>α-smooth muscle actin-FITC</td>
<td>mouse, mc</td>
<td>1:250</td>
<td>Sigma Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>tubulin</td>
<td>mouse, mc</td>
<td>1:1000</td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td>Protein A/G PLUS-Agarose (IP)</td>
<td></td>
<td></td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td>rabbit IgG</td>
<td>goat</td>
<td>1:2500</td>
<td>New England Biolabs (Beverly, MA, USA)</td>
</tr>
<tr>
<td>mouse IgG</td>
<td>goat</td>
<td>1:2500</td>
<td>Upstate (Charlottesville, VA, USA)</td>
</tr>
</tbody>
</table>

Table VIII: antibodies and beads

Antibodies were diluted in TBST-T, 5 % milk or 5 % BSA according to the recommendation of the provider.

*Materials and Methods* – 41 –
5. ROS detection

5.1. Intracellular ROS detection with FACS

Intracellular ROS was detected using 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA). This dye is a non-fluorescent compound, which is able to cross the membrane and enter the cell. Once in the cell the cellular esterase cleaves the dye producing 2’,7’-dichlorofluorescein (H$_2$DCF) which is unable to pass membranes again. This form can be easily oxidized by intracellular ROS leading to the fluorescent product 2’,7’-dichlorofluorescein (DCF) and monitored by flow cytometry (FACS).

In 6-well plates 0.25 x 10$^6$ cells/well were seeded and grown for 24 h, then starved with DMEM (0.1 % FCS) for additional 24 h. Cells were washed with warm HBSS-buffer (37 °C), then HBSS was added to each well followed by preincubation with inhibitors (RV, DPI, NAC) for indicated times. As vehicle control 0.1 % DMSO was used. After 15 min cells were loaded with 20 μM H$_2$DCF-DA final concentration per well. Cells were stimulated after additional 15 min and then the buffer was aspirated, cells were trypsinized

<table>
<thead>
<tr>
<th>Solutions and buffers for ROS measurement</th>
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</thead>
<tbody>
<tr>
<td><strong>Solutions</strong></td>
</tr>
<tr>
<td>FACS buffer pH 7.37</td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>PBS/BSA 2%</th>
<th>BSA dissolved in PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS Buffer</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
</tr>
<tr>
<td></td>
<td>Na$_2$HPO$_4$</td>
</tr>
<tr>
<td></td>
<td>KH$_2$PO$_4$</td>
</tr>
<tr>
<td></td>
<td>CaCl$_2$ x H$_2$O</td>
</tr>
<tr>
<td></td>
<td>MgSO$_4$</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
</tr>
<tr>
<td></td>
<td>ddH$_2$O</td>
</tr>
</tbody>
</table>

Table IX: ROS detection solutions
and the reaction was stopped with 2% BSA in PBS. For detection an emission wavelength of 525 nm on the fluorescence channel FI1h was measured using a FACSCalibur™ (BD). The peaks were recorded at the FACS and geometric mean was analysed using CellQuest™ Pro software.

5.2. Extracellular ROS detection using Amplex Red™ Assay

The assay was performed by Mario Kumerz (Doctoral student in our group) using the method described in his thesis (Mai 2009, Uni. Vienna). The non-fluorescent compound Amplex Red™ (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen, CA, USA) was used which is a substrate of horseradish peroxidase (HRP) and is able to be converted into resoruflavin in the presence of H₂O₂ in a 1:1 stoichiometry.

In brief, VSMC were grown in 24-well plates and serum-starved for 24 hours. Cells were washed once with prewarmed PBS before the KRPG buffer containing the Amplex Red™ reagent was applied to the cells. Plates were incubated for 15 minutes at 37°C before VSMC were stimulated with different compounds for 5 - 15 minutes. 80 μl of the supernatant was transferred into 96-well plates and produced resoruflavin was measured in triplicates with a fluorometer (Genios Pro, Tecan, Switzerland).

6. Oxidized PTP detection assay

To detect the oxidation of PTPs in cells a special protocol based on antibody against oxidized PTPs was used. First all reduced PTPs have to be protected efficiently with the help of iodoacetic acid (IAA) to prevent a pervanadate mediated oxidation. Only the already oxidized PTPs should be prone for the later processing of the probes. Then the oxidized PTPs are reduced by treating the samples with dithiothreitol (DTT) to make them susceptible to the later incubation with pervanadate. The pervanadate is converting the PTP from a sulfinic form to a sulfonic acid form, which is an irreversible oxidized form. This sulfonic form can be detected with a specific antibody directed against the conserved motif VHCSAG of PTPs, containing the active-site cysteine after western blotting of the proteins.
Figure 9: Antibody-based oxidized PTP detection assay
Schematic overview of the method to detect oxidized PTPs with a specific antibody. After stimulation all reduced PTPs are protected for further oxidation by IAA treatment. Only oxidized PTPs are then reduced with DTT and converted from a sulfenic to an irreversible oxidized sulfonic acid form using pervanadate. The sulfonic form can be detected after western blotting of the probes with a specific antibody. Adopted from Persson C. et al\textsuperscript{197}.

3.5 x 10\textsuperscript{6} VSMC were seeded in a 15 cm dish, grown over night and starved with DMEM (0.1% CS) for 24 h. Prior to the experiment DMEM without supplements was added to the cells and then the cells were stimulated as described. The lysis buffer was degassed with a water-jet vacuum pump for 30 – 45 min and to half of the buffer IAA (100 mM final concentration) was added, since IAA has to be protected from light during the following processing the samples were sheltered from light. The non-IAA treated samples represent the positive controls, reflecting the total amount of possible oxidized PTPs in the probes. Therefore always two sample were stimulated the same way, one was lysed with IAA the other one without IAA in the lysis buffer. Before adding 750 μl lysis buffer to the dishes the cells were washed twice with ice cold PBS. After 10 min incubation of the cells with lysis buffer they were scrapped off and pelleted by centrifugation at 13,000 rpm (10 min at 4 °C). To the supernatant a phosphatase specific antibody (anti-Shp2) was added (1.5 μg) and incubated for 2 hours at 4 °C followed by in incubation with 30 μl Protein A/G PLUS-Agarose beads (Santa Cruz) at 4 °C for one hour. The beads were spun down briefly and then washed once with 500 μl lysis buffer and 3 times with 500 μl degassed 20 mM Heps buffer. To reduce the sulfenic form of oxidized cysteins in the PTP acitive site 100 mM DTT (100 μl/probe) was added to the samples for 30 min and then beads were washed again three times with Heps buffer. After that, lysates were treated with freshly prepared Pervanadate (100 mM) at a final concentration of 100 μM for 1 h. Samples were

\[ \text{H}_2\text{O}_2, \text{PDGF, UV, AngII?} \]

\[ \text{glutathione} \]

\[ \text{oxidized} \]

\[ \text{S} \]

\[ \text{S-} \]

\[ \text{O}_3\text{-H} \]

\[ \text{Reduced} \]

\[ \text{S-} \]

\[ \text{CH}_2\text{COOH} \]

\[ \text{Oxidized} \]

\[ \text{PTPs} \]
briefly spun at 2000 rpm, the supernatant was discarded and 20 μl 3xSDS sample buffer was added to the beads and heated to 95 °C for 5 min. The probes were stored at -80 °C until processing by SDS-PAGE and western blotting. For the detection of the oxidized PTPs an Oxidized PTP active site antibody (R&D systems) was used at a concentration of 1 μg/ml. The primary antibody was applied over night, the secondary for 2 h at room temperature with TBS-T washing between primary and secondary antibodies and before detection.

7. siRNA knock down in VSMC

7.1. Transfection of VSMC

For knocking down a specific protein VSMC were transfected with siRNA. 0.5 x 10^6 cells were seeded in 6 cm dishes. 5 h after seeding the cells they were transfected, using siRNA specific against Nox4 (ThermoFisher Scientific) or a Stealth™ siRNA kit (Invitrogen) specific for Shp2 (rat PTPN11). As scrambled control, Negative control med GC (Invitrogen) was used. Oligofectamin (Invitrogen) was dissolved in warm Opti-mem® I (Gibco), mixed by vortexing and left at room temperature for 5 min. The siRNA was diluted to a final concentration of 50 μM in warm Opti-mem® I and then the siRNA and Oligofectamine were mixed by pipetting up and down and incubated at room temperature for 20 min. Before adding the siRNA to the cells they were washed twice with Opti-mem® I and 1.67 ml of it were put on each well. Then 330 μl of the siRNA/Oligofectamine mix were added drop

### siRNA Sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| **Nox4:** | 5'-ACUGAGGUACAGCUGGAUGUU-3'  
5'-CAUCCAGCUUGCACUCGU-3' | sense  
anti-sense |
| **Shp2:** | 5'-UACUUGACACACUUGCUUCCUCUCUC-3'  
5'-GAGGGAAGAGCAGCAUGUGUCAAGUA-3'  
5'-GGACUGUGACAGCGCUUCCUCUAAA-3'  
5'-UUUAGGAACGUGAGCAGCUUCCUCUAAA-3'  
5'-UCCACCAGGGAAUACUUAAUUGG-3'  
5'-CCAAUAUAAGUAAUCCCCUGGUGGA-3' | sense  
anti-sense  
anti-sense  
anti-sense  
anti-sense  
anti-sense |

Table XI: siRNA sequences
wise to each dish. The siRNA was left on the cells over night, the next day it was replaced by DMEM (10 % CS). For further experiments the cells were used 72 h after transfection with siRNA. Prior to an experiment the cells were starved with DMEM (0.1 %FCS) for 24 h.

8. RNA isolation and PCR

8.1. RNA isolation

VSMC were grown in 6 cm dishes and for the RNA isolation two dishes were pooled. Total RNA was isolated using peqGOLD total RNA Kit (Peqlab) according to the manufacturer’s protocol. Cells were lysed with provided lysis buffer and homogenized with the supplied shredder columns. Before applying the lysates to the binding columns they were mixed with 70 % ethanol. After RNA binding to the columns they were washed and DNA was digested by on-column treatment with DNase. Again the columns were washed, dried and RNA was eluted in 50 μl RNase-free water. The quality of the RNA was controlled on a 1 % -agarose gel and by measuring the RNA concentration at 260 nm with a spectrophotometer (BioPhotometer; Eppendorf AG).

Buffers for RNA gel

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Reagents</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10x TBE</td>
<td>Tris base</td>
<td>108 g</td>
</tr>
<tr>
<td></td>
<td>Boric acid</td>
<td>55 g</td>
</tr>
<tr>
<td></td>
<td>0.5 M EDTA pH 8.0</td>
<td>40 ml</td>
</tr>
<tr>
<td></td>
<td>ddH2O</td>
<td>ad 1000 ml</td>
</tr>
<tr>
<td>10x sample buffer</td>
<td>Bromphenol blue</td>
<td>4.0 mg</td>
</tr>
<tr>
<td></td>
<td>ddH2O</td>
<td>5.0 ml</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Agarose gel 1 %</td>
<td>Agarose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>SYBR Safe DNA gel stain</td>
<td>10,000 x conc (in DMSO)</td>
<td>added after boiling of the agarose</td>
</tr>
</tbody>
</table>

Table XII: Buffers for RNA gel

---

Materials and Methods
Materials and Methods

The quantity is correlated to the absorbance at 260 nm and can be calculated using the following formula:

\[ \text{RNA concentration (\( \mu g/mg \)) = \text{Absorbance}_{260} \times 40 \times \text{dilution factor}} \]

8.2. Reverse transcription

To analyse the RNA with the PCR technique RNA was converted to a complementary-DNA (cDNA). For this purpose Superscript™ II Reverse Transcriptase (Invitrogen) and reagents provided with the Superscript™ First-Strand Synthesis System (Invitrogen) were used. The RT reaction was performed using 2 to 5 \( \mu g \) RNA, 10 ng random hexamers/\( \mu g \) RNA, 1 \( \mu l \) 10 mM dNTP mix (deoxyribonucleotides), 1 \( \mu l \) luciferase RNA (10^8 copies, exogenous standard) and filled to a total volume of 12 \( \mu l \) with RNase-free water. Samples were incubated at 65 °C for 5 min for denaturation, left on ice for 1 - 2 min and 4 \( \mu l \) 5x first strand buffer, 2 \( \mu l \) DTT (0.1 M) and 1 \( \mu l \) RNaseOUT inhibitor (40 U/\( \mu l \)) were added. This mix was left at room temperature for 2 min in order to allow primers to anneal. Then 1 \( \mu l \) Superscript™ II RT enzyme (50 U/\( \mu l \)) was added. After incubated at 42 °C for 60 - 90 min the enzyme was inactivated by heating the samples to 70 °C for 15 min. To digest remaining RNA 1 \( \mu l \) RNase H was added to each tube for digesting remaining RNA. Finally the cDNA was purified using Bio-Spin® 30 Tris Columns (BIO-RAD). Columns were dried by centrifugation at 1,000 g for two min to remove packing buffer. The cDNA was loaded on the resin drop wise and spun at 1,000 g for 4 min. cDNA was stored at -20 °C, for short time storage kept at 4 °C.

8.3. Quantitative real-time PCR

To quantify the expression level of a specific mRNA a quantitative real-time polymerase chain reaction (qPCR) was performed. For this purpose two gene-specific primers (forward and reverse) were used. Amplification of DNA was detected using a LightCycler™ LC480 (Roche Diagnostics) system which detects fluorescence of the dye SYBR® Green. This dye can only bind to double stranded DNA by intercalating with the minor groove of the DNA double helix. During the amplification, the DNA amount increases exponentially, therefore also the signal increases the same way.
The real-time PCR was carried out using LightCycler™ LC480 SYBR Green I Master reagent (Roche Diagnostics). For target gene detection cDNA was diluted in RNase-free water 1:4, for the detection of the housekeeping gene (18 S RNA) a dilution of 1:1000 was made. The reaction mix contained 5 μl SYBR Green Master Mix, 0.5 μl primer solution (7.5 μM) for each primer and 8.5 μl PCR-grade water. 14 μl of the mixture was put into each well of a PCR plate and 1 μl cDNA was added, followed by a brief spin at 2,000 rpm. PCR was performed according to the manufacturer’s protocol and data were analysed using LightCycler™ LC480 software.

9. Statistical analysis

All statistic analyses were performed with GraphPad PRISM™ software (GraphPad Software Inc). Data were normalized using stimulated samples as 100 % values. When comparing multiple treatment groups a one-way ANOVA combined with a Dunnett post test was accomplished. For comparison of two groups a two-tailed paired t-test was used. Values with p< 0.05 were considered as significant and all graphs show ±SEM.
D Results

1. Characterisation of VSMC

Subcultured VSMC develop a proliferative phenotype resembling the cells in the neointima. As shown by our group previously, Ang II induces hypertrophy in VSMC which could be inhibited by RV. The proposed mechanism was that RV activates Shp2, thus preventing interaction between Gab1 and PI3K that is necessary for the further activation of Akt. To further characterize the mechanism of this RV-mediated inhibition of Ang II signaling, we first isolated VSMC from rat thoracic aortas as described in Material and Methods.

Smooth muscles have the unique ability to contract and relax in response to changes in the environment surrounding them. The so called “contractile actin” is important for the dynamic remodelling process. Therefore after isolation the identity of the VSMC was confirmed by immunostaining with a FITC-conjugated α-smooth-muscle-actin antibody. By labelling specifically α-actin we could show the lattice-like structure of the actin filament systems (Fig.10).

*Figure 10: Anti-α-smooth muscle actin staining of VSMC*
Image shows VSMC immunostained with FITC-conjugated anti-α-smooth muscle actin antibody in 400-fold magnification.
2. RV inhibits Akt phosphorylation upon Ang II and EGF stimulation

Previous publications showed that RV can specifically inhibit Ang II- or EGF-induced Akt phosphorylation when preincubated with VSMC 30 min prior to stimulation.\textsuperscript{42, 44} We therefore first of all verified this effect in our experimental settings. Preincubation of cells with 50 μM RV for 30 min followed by stimulation with either Ang II (100 nM) for 10 min (Fig. 11A) or EGF (100 ng/ml) for 5 min (Fig. 11B) led to a diminished phosphorylation of Akt. Ang II stimulation normally led to a 2-4 fold increase of Akt phosphorylation, EGF treatment could elevate Akt phosphorylation even 10-fold.

![Figure 11: RV reduces Ang II- or EGF-induced phosphorylation of Akt](image)

Quiescent VSMC were pretreated with 50 μM RV for 30 min and then stimulated with 100 nM Ang II (A) for 10 min or 100 ng/ml of EGF for 5 min (B). Cells were lysed and protein detection was performed by western blot analysis using antibodies against pAkt and as loading control against tubulin. As vehicle control 0.1 % DMSO was used. One representative blot is shown.

3. Putative redox-related action of RV

3.1. Inhibition of intracellular and extracellular ROS by RV treatment in VSMC

RV is well know for its antioxidative effects\textsuperscript{200} and therefore one aim of our study was to investigate whether RV acts as an antioxidant when inhibiting Akt phosphorylation. By inhibiting ROS, RV could lead to a restoration of the Shp2 activity by protecting it from oxidation explaining how RV is able to activate Shp2.\textsuperscript{44} A suggested mechanism for
the antioxidative effect of RV would be a modulation of Noxes, potential ROS sources in VSMC. Therefore we used dihydroxyiodinium (DPI) as an unspecific inhibitor of flavin-containing enzymes including NADPH oxidases, xanthine oxidases and cytochrome P450 enzymes\textsuperscript{201} as positive control to reduce ROS and to compare its impact with the effect of RV. It has been shown before that Ang II stimulation of VSMC leads to ROS production that contributes to VSMC-growth by activation of the p38MAPK cascade downstream of the AT1 receptor\textsuperscript{186} or by transactivation of the EGF-R through Nox dependent ROS\textsuperscript{90, 95}.

To test, whether RV can inhibit Ang II- or EGF-mediated ROS production we loaded the cells with 20 μM H\textsubscript{2}DCF-DA, a dye which can be easily oxidized by ROS in the cell leading to a fluorescent product, which can be measured with FACS for detection of intracellular ROS. Ang II stimulation for 15 min was able to induce ROS production double-fold compared to the vehicle (DMSO) control. RV pretreatment was able to reduce ROS production nearly to basal levels (Fig 12\textsuperscript{A}) and also preincubation with DPI, the positive control, inhibited ROS in accordance to literature.\textsuperscript{202} In contrast, treatment of the cells with 100 ng/ml EGF for 10 min did not lead to a significant activation of intracellular ROS production in comparison to the vehicle control (0.1 % DMSO). However, RV and DPI were able to inhibit the intracellular ROS levels by about 50 % (Fig. 12\textsuperscript{B}), again verifying a strong antioxidant effect for both compounds.

\textbf{Figure 12: RV diminishes intracellular ROS level after Ang II and EGF stimulation}  
Quiescent VSMC were incubated 15 min with 20 μM H\textsubscript{2}DCF-DA in HBSS buffer, then they were preincubated with 50 μM RV or 0.1 % DMSO (vehicle control) for 30 min or 10 μM DPI for 1 h and finally stimulated with 100 nM Ang II for 15 min (\textit{A}) or with 100 ng/ml EGF for 10 min (\textit{B}). ROS production (equivalent to the amount of formed DCF) was analysed by flow cytometry. Graphs show average fluorescent signal of three independent experiments. Ang II or EGF stimulation was set to 1 (**, p < 0.01; ns, not significant; one-way ANOVA versus Ang II-or EGF-treatment).

\textit{Results} – 53 –
Upon EGF stimulation it was shown that \( \text{H}_2\text{O}_2 \) was produced extracellular after EGF-R transactivation, also influencing phosphatases. Examining extracellular \( \text{H}_2\text{O}_2 \) production in VSMC we applied an Amplex Red\textsuperscript{TM} assay (performed by Mario Kumerz, Dissertation 2009, Uni. Vienna), which is detecting extracellular produced \( \text{H}_2\text{O}_2 \).

In accordance with our intracellular ROS data we also observed a high level of extracellular \( \text{H}_2\text{O}_2 \) produced by unstimulated VSMC. Stimulation with Ang II and EGF was able to induce this production of extracellular \( \text{H}_2\text{O}_2 \), in a time-dependent manner (Fig. 13 A-B). EGF treatment led to a peak of \( \text{H}_2\text{O}_2 \) after 15 min (20 % above basal level). \( \text{H}_2\text{O}_2 \) production was highest 10 min after Ang II stimulation (15 % above basal level) but declined at later time points.

**Figure 13:** RV extenuates both basal and Ang II- or EGF-induced extracellular \( \text{H}_2\text{O}_2 \) levels

(A) Serum starved VSMC were stimulated in Amplex Red buffer with EGF (100 ng/ml) for 5 to 15 minutes after preincubation with RV or vehicle control for 30 minutes. \( \text{H}_2\text{O}_2 \) production was quantified using a fluorometer. Absolute values were correlated to catalase-treated cells used as negative control. Graph shows relative extracellular \( \text{H}_2\text{O}_2 \) production expressed as x-fold vehicle-treated cells (–□–, vehicle-treated cells; –■–, RV-treated cells; ***, \( p < 0.001; \text{mean} \pm \text{SEM; } n = 4 \)). (B) Cells were stimulated with Ang II (100 nM) and then treated and analyzed as indicated in (A). (–□–, vehicle-treated cells; –■–, RV-treated cells; ***, \( p < 0.001; \text{mean} \pm \text{SEM; } n = 4 \)). For all statistical analyses, vehicle-treated cells were correlated to RV-treated cells for each time point separately.

*Experiment was performed by Mario Kumerz (Doctoral student in our group, Thesis May 2009, Uni. Vienna).*
3.2. Role of Noxes in Ang II- and EGF-signaling in VSMC

RV acts as antioxidant in our cells as it was able to inhibit intra- and extracellular ROS levels. One major ROS source in VMSC are Noxes and therefore a suggested mechanism of RV-mediated ROS inhibition could be via influencing Noxes. In addition, RV has been shown to suppress Ang II-induced Nox activity in endothelial cells (EC). Nox 1 and Nox4 are the two Nox proteins expressed in VSMC. Both have been shown to play a role in mitogenesis and hypertrophy in the cardiovascular system. Nox4 was detected in focal adhesions of VSMC where also the EGF-R is localized after tyrosine phosphorylation. In Ang II signaling in VSMC ROS production by Nox1 is suggested as the major component for the transactivation of the EGF-R, whereas the role of Noxes downstream of the EGF-R as well as the role of Nox4 in Ang II signaling is unclear.

3.2.1. Role of Nox4

3.2.1.1. Specific knock down of Nox4 mRNA in VSMC using siRNA

To investigate whether Nox4 is important for the signaling after EGF or Ang II stimulation towards major kinases (Akt, p38, ERK1/2) Nox4 was inhibited by treatment with 50 µM siRNA for 72 h. By performing real-time PCR, the level of Nox4 mRNA was analyzed and a decline of Nox4 mRNA levels to 15 % compared to scrambled control treated cells.

![Figure 14: Specific down-regulation of Nox4 in VSMC](image)

Knock down was achieved by treating the cells with 50 µM siRNA against Nox4 or 50 µM scrambled control for 72 h. mRNA was isolated and expression of Nox4 was detected with real-time PCR. Graphs show mean Nox4 mRNA (A) or Nox1 mRNA (B) level relating to 18S mRNA levels of three independent experiments. Data were normalized setting scrambled control to 1 (***, p < 0.001; t-test).
was detected (Fig. 14A). In contrast, the mRNA level of Nox1 was not impaired by Nox4 siRNA treatment (Fig. 14B), indicating a specific down-regulation of Nox4 mRNA by the used siRNA.

### 3.2.1.2. Nox 4 knock down does not influence phosphorylation of Akt after Ang II stimulation nor inhibition by RV

To test the influence of Nox4 knock down on the phosphorylation levels of several kinases in VSMC and on the RV-mediated inhibition, cells were treated with Nox4 siRNA (50 μM) or scrambled control siRNA (50 μM) for 72 h, then cells were stimulated as indicated and protein levels of pAkt, pp38 and pERK1/2 were detected by western blotting. Knock down of Nox4 had no impact on the phosphorylation of Akt after Ang II (100 nM, 10 min) stimulation, and also the inhibitory effect of RV (50 μM, 30 min) on phosphorylated Akt was not influenced (Fig. 15A). The phosphorylation level of p38 (Fig. 15B) and ERK 1/2 was detected (Fig. 14A). In contrast, the mRNA level of Nox1 was not impaired by Nox4 siRNA treatment (Fig. 14B), indicating a specific down-regulation of Nox4 mRNA by the used siRNA.

![Figure 15: Ang II and RV-mediated inhibition of Akt, p38 and ERK1/2 phosphorylation is independent of Nox4](image-url)

Serum-deprived Nox4 siRNA-treated cells were pretreated with RV (50 μM, 30 min) or 0.1 % DMSO followed by stimulation with 100 nM Ang II for 10 min. Phosphorylation of Akt (A), p38 (B) and ERK (C) was detected by western blot analysis. Densitometric analysis was performed; mean protein levels of three independent experiments are shown in the graphs. Ang II stimulation of scrambled control was set to 100 % (**, p < 0.01; *, p < 0.05; ns, not significant; one-way ANOVA versus Ang II-treatment).
(Fig. 15C) was comparable to that of scrambled control treated cells. This excludes Nox4 as a major source of ROS during Ang II stimulation by showing that Nox4 is not needed for the phosphorylation of Akt, p38 or ERK1/2. Furthermore RV seems not to act as a specific inhibitor of Nox4 as the knock down can not mimic the effect of RV on Akt phosphorylation nor blunt the inhibitory effect of RV.

3.2.1.3. VSMC with a Nox4 knock down show unaltered Akt phosphorylation in response to EGF treatment

Next the effect of EGF stimulation on Nox4 siRNA treated cells was investigated. Cells which were impaired in Nox4 expression by siRNA were pretreated with RV (50 μM, 30 min) and stimulated with EGF (100 ng/ml, 5 min). Akt phosphorylation (Fig. 16A) as well as phosphorylation of p38 (Fig. 16B), and ERK1/2 (Fig. 16C) were analyzed by

Figure 16: EGF-induced phosphorylation of Akt, p38 and ERK1/2 is not influenced by knock down of Nox4 with siRNA

Nox4 diminished cells were serum-starved and then activated with 100 ng/ml EGF for 5 min after incubation with RV (50 μM) or 0.1 % DMSO for 30 min. Proteins were detected by western blotting with antibodies against pAkt (A), pp38 (B), and pERK1/2 (C). Tubulin was used as loading control. Graphs represent results from densitometric analysis of three independent experiments. EGF stimulation of scrambled control was set to 100 % (**, p < 0.01;*, p < 0.05; ns, not significant; one-way ANOVA versus EGF-treatment).
western blotting. EGF stimulation led to activation of all three kinases comparable to the phosphorylation in the scrambled control cells which was inhibited by RV. Only the inhibition of ERK1/2 phosphorylation by RV was slightly diminished by Nox4 knock down.

These data indicate that Nox4 does not play a major role in Ang II- or EGF-induced MAPK and Akt phosphorylation in VSMC. Thus, Nox4 is no suggestive target of RV.

3.2.2. Role of Nox1

3.2.2.1. Nox1 suppression abolishes Ang II stimulation of Akt and p38

We next investigated, whether Nox1 is involved in the RV-mediated modulation of Akt signaling. This could be mediated by Rac, an important subunit for Nox1 activation which has been shown to be downregulated by RV in breast cancer cells\(^ {206}\). To inhibit Nox1 activation we applied a blocking peptide (gp91ds-tat) to the cells. The peptide consists of nine amino acids from p91phox that normally binds to p47phox. Originally, the peptide was designed to inhibit Nox2 assembling, but it was also shown to inhibit Nox2 homologues in the vascular system.\(^ {172}\) With this p91phox docking sequence (ds) the blocking peptide inhibits the assembling of Nox1 with regulatory proteins by interfering with the p47phox binding by acting as decoy that binds p47phox\(^ {180,207}\). The tat-sequence is needed for import into the cells. As the recruitment of p47phox is important for the activation of Nox1 but not Nox4, the peptide could be used as a specific Nox1 inhibitor in VSMC\(^ {169}\). As control, a nonsense peptide of the same length with a tat-sequence was used (gpco-tat).

Cells were pretreated with 50 μM RV, 100 μM gp91ds-tat or gpco-tat protein for 30 min, then stimulated as previously described with Ang II. Cells were lysed and phosphorylation of kinases was detected by western blot analysis. Gp91ds-tat-pretreatment led to an inhibition of Ang II-mediated Akt phosphorylation, while in control peptide-treated cells detected Akt phosphorylation was not influenced (Fig. 17A-B). In contrast to RV, gp91ds-tat peptide inhibited p38 phosphorylation significantly by more than 50 % (Fig. 17C) but phosphorylation of ERK only showed at slight but not significant trend of reduction (Fig. 17D). The control peptide did not influence the phosphorylation of Akt, p38 and ERK (Fig. 17A).
Figure 17: Nox1 inhibition blunts the phosphorylation of Akt and p38 induced by Ang II
Quiescent VSMC were pre-treated with 100 µM gp 91 blocking peptide or control peptide, 50 µM RV or vehicle control for 30 min, then stimulated with 100 nM Ang II for 10 min. Western blot analysis was performed, one representative blot is shown (A). Graphs represent mean densitometric signal of three independent experiments staining pAkt (B), pp38 (C), pERK (D). Ang II stimulation is set to 100 % (**, p < 0.01; *, p < 0.05; ns, not significant; one-way ANOVA versus Ang II-treatment).

3.2.2.2. Nox1 inhibition does not alter the response of kinase phosphorylation upon EGF stimulation

We then aimed to clarify the dependence of the signaling downstream of the EGF-R on Nox1 in our cell system. Cells were incubated with RV, gp91ds-tat peptide or control peptide for 30 min, and then activated with 100 ng/ml EGF for 5 min. Subsequently the phosphorylation of Akt, p38 and ERK was detected by western blotting. In contrast to stimulation with Ang II, EGF-induced phosphorylation of all tested kinases was not significantly influenced by Nox1 inhibition, although graphs might show a tendency of kinase inhibition especially regarding p38 and ERK1/2 (Fig. 18A-D). Again, the control peptide did not inhibit any kinase phosphorylation significantly. This proofs an involvement of Nox1 for the Ang II signaling but indicated that downstream of the EGF-R no Nox1 or Nox4 activity is necessary for activation of Akt, p38 and ERK.
Figure 18: Phosphorylation of Akt, p38 and ERK after EGF stimulation is independent of Nox1

Incubation of quiescent cells with 100 μM gp 91 blocking peptide or gp co control peptide, 50 μM RV or vehicle control for 30 min was followed by treatment with 100 ng/ml EGF for 5 min. Protein amount was detected by western blot analysis. For each kinase one representative blot is shown. Mean protein levels measured by densitometric analysis of three individual experiments are shown. Data were normalized setting EGF stimulation to 100 % (*, p < 0.05; ns, not significant; one-way ANOVA versus EGF-treatment).

3.2.2.3. Nox inhibitor AEBSF inhibits ROS production after Ang II stimulation but not Akt phosphorylation

To further verify whether the inhibition of Akt phosphorylation could be due to inhibition of Noxes by RV, we used as an alternative Nox blocker\textsuperscript{208}. AEBSF is a serine protease inhibitor that blocks the recruitment of p47phox to the Noxes enzymes\textsuperscript{172} and can be therefore used as Nox1 inhibitor.

Cells were treated with AEBSF for 30 min, then loaded with 20 μM H\textsubscript{2}DCF-DA and subsequently stimulated with Ang II for 15 min. Intracellular ROS was measured by flow cytometry. We found that 100 μM AEBSF inhibited intracellular ROS significantly (Fig. 19).
Next we asked if AEBSF can impede the phosphorylation of Akt similar to RV. Treatment of VSMC with 10-200 μM AEBSF for 30 min as used in mouse fibroblasts\textsuperscript{209} or 2 h before stimulation with Ang II for 10 min showed, that it was not able to block the Akt signal (Fig. 20A), although 100 μM were able to abolish ROS induction (Fig. 19). Treating the cells with up to 1 mM AEBSF as used in mesengial cells\textsuperscript{210}, the phosphorylation of Akt was even activated, whereas p38 and ERK phosphorylation were inhibited at these high concentrations (Fig. 20B-C). Taken together these data show an effect of AEBSF influencing Ang II-induced ROS in our cell system, whereas its influence on the phosphorylation of Akt, p38 and ERK1/2 are contrarious as higher dosage increased Akt phosphorylation but seemed to inhibit phosphorylation of p38 and ERK.

3.3. Inhibition of Ang II- but not EGF-induced phosphorylation of Akt by DPI and NAC

It was previously demonstrated that the inhibitory effect of RV on VSMC hypertrophy is mediated mainly by interfering with phosphorylation of Akt\textsuperscript{42} whereas other MAPK (p38/ERK1/2) remain largely unaffected. Our previous experiments indicated at role of Nox1 for Ang II-induced Akt phosphorylation but neither Nox1 nor Nox4 seemed to be important for the EGF-mediated signaling. To clarify the dependence of Ang II or EGF stimulation on ROS in our cells we applied two antioxidative substances, the flavoprotein inhibitor DPI and a general antioxidant and precursor of glutathione N-acetyl cysteine (NAC), on the cells.

**Figure 19: AEBSF treatment decreases intracellular ROS after Ang II stimulation**

Serum-starved VSMC were preincubated for 30 min with AEBSF (50 μM, 100 μM, 200 μM), incubated 15 min with 20 μM H\textsubscript{2}DCF-DA in HBSS buffer and then cells were stimulated with 100 nM Ang II for 15 min. Using flow cytometry intracellular ROS was measured. Graphs show average fluorescent signal from three independent experiments. Ang II stimulation was set to 1, (*, p < 0.05; one-way ANOVA versus Ang II-treatment).
Figure 20: AEBSF has no influence on Ang II-induced phosphorylation of Akt
Quiescent cells were loaded with 10 - 1000 μM AEBSF for 30 min or 2 h and activated with 100 nM Ang II for 10 min followed by western blot analysis using antibodies against pAkt (A), pp38 (B), pERK1/2 (C) and tubulin as loading control. Graphs show the mean densitometric signals of pAkt (A), pp38 (B) and pERK1/2 (C) antibodies detected after 30 min AEBSF preincubation time. One representative blot is shown for pAkt (A) with AEBSF pretreatment for 30 min or 2 h. Graphs represent densitometric signal, measured in one to three experiments, Ang II stimulated cells were set to 1.

We pretreated VSMC with 10 μM DPI for 1 h or 10 mM NAC for 2 h and cell lysates were subjected to Western blot analysis using antibodies against phospho-Akt, phospho-p38, and phospho-ERK and for normalization against tubulin. Blocking of Akt phosphorylation after Ang II stimulation with DPI and NAC was already shown in VSMC. We found that Ang II (100 nM, 10 min) induced phosphorylation of Akt was blocked by NAC preincubation (Fig. 21A) as effective as with RV while DPI inhibited Akt phosphorylation was less efficient. Phosphorylation of Akt upon EGF stimulation (100 ng/ml, 5 min) could only be selectively inhibited by RV pretreatment. NAC and DPI preincubation did not interfere with phosphorylation of Akt when induced by EGF stimulation (Fig. 21B). RV had no effect on the phosphorylation of p38 after Ang II stimulation whereas NAC and DPI were able...
Figure 21: Antioxidants inhibit Ang II-induced phosphorylation of Akt, p38 and ERK
Serum-deprived cells were pretreated with 50 μM RV or DMSO for 30 min, 10 μM DPI for 1 h or 10 mM NAC for 2 h, then stimulated with 100 nM Ang II for 10 min and lysates were subjected to western blot analysis using antibodies to pAkt (A-B), pp38 (C-D) and pERK1/2 (E-F). Tubulin served as loading control. For each antibody one representative blot is shown, graphs represent densitometric signal obtained by three independent experiments. Ang II or EGF stimulation was set to 100 % (**, p < 0.01;*, p < 0.05; ns, not significant; one-way ANOVA versus Ang II-or EGF-treatment).
to block the phosphorylation to basal levels (Fig. 21C). In contrast both antioxidants had no effect on p38 phosphorylation after EGF stimulation (Fig. 21D). Phosphorylation of the MAPK ERK1/2 was not significantly inhibited by RV pretreatment after Ang II stimulation, only NAC could block Ang II-induced ERK 1/2 phosphorylation by about 50% (Fig. 21E). In the EGF stimulated cells no effect of NAC or DPI on the phosphorylation of ERK1/2 could be detected (Fig. 21F). These results indicate that antioxidants such as NAC and DPI can mimic the effect of RV on Ang II-mediated pAkt, but not on EGF-mediated pAkt.

3.4. Effects of additional antioxidants on VSMC

We showed before that phosphorylation of different kinases upon stimulation with Ang II could be blocked by two different general antioxidative active compounds in VSMC and is most likely redox dependent. To verify these results, other known redox-active compounds with a more selective mode of action were tested in a similar experiment-setup.

3.4.1. Hydroxyl radical scavengers do not interfere with phosphorylation of Akt in VSMC

Two potential membrane crossing hydroxyl radical (OH·) scavengers N-(2-mercaptpropionyl)-glycine (N-MPG) and dimethyl thiourea (DMTU) were tested whether they could influence the Ang II-induced Akt signaling in VSMC as RV does. MPG is cell-permeable and a powerful scavenger of OH·. DMTU is highly diffusible, has a long half-life and high potency in scavenging hydrogen peroxide (H₂O₂) and OH·. VSMC were preincubated with 50 μM to 10 mM DMTU for 30 min or 2 h before stimulation with Ang II as previously described. DMTU pretreatment did not affect the Akt, p38 or ERK1/2 phosphorylation induced by Ang II (Fig. 22A). Pretreatment of cells with 50 – 300 μM MPG for 30 min (Fig. 22B) showed a slight inhibition of phosphorylation of Akt and p38 after Ang II stimulation detected by western blot analysis. But as this effect was not always reproducible and therefore not statistically significant when repeating the experiments several times we did not pursue this effect any longer.
Figure 22: Hydroxyl scavengers cannot inhibit Akt phosphorylation in Ang II-activated VSMC

Serum-deprived VSMC were pretreated with 50 μM - 10 mM DMTU (A) or 50 to 300 μM MPG (B) for 30 min or 2 h, and then stimulated with 100 nM Ang II for 10 min. Proteins were subjected to western blot analysis using antibodies to pAkt, p38 and pERK1/2 as well as tubulin as loading control. One representative blot is shown from one to three individual experiments.

3.4.2. No effect of SOD and catalase mimetics on phosphorylation of Akt

Two main intracellular defence mechanisms against ROS are superoxide dismutase (SOD) and catalase. SOD dismutates O$_2^•$ to H$_2$O$_2$ and catalase is catalysing the reaction of H$_2$O$_2$ to water. Beside inhibition of Noxes RV could also decrease ROS by detoxifying it a way similar to SOD and catalase action in the cells. Therefore two SOD and catalase mimicking compounds were tested in VMSC. EUK 134 and Mn(III)terakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnIIITM), both manganese-porphyrins, were used with 30 min or 2 h preincubation time in concentrations of 50-200 μM, according to the literature. When cells were stimulated with Ang II neither of these two compounds was able to interfere with the Akt phosphorylation detected by immunoblotting (Fig. 23 A-B).

These data indicate that selective inhibition of O$_2^•$, H$_2$O$_2$, and OH’ formation cannot lead to inhibition of phosphorylation of Akt upon Ang II stimulation, and that it is therefore unlikely that RV interferes with phosphorylation of Akt by detoxification of a specific form of reactive oxygen species.

Results
Figure 23: Phosphorylation of Akt is not impeded by SOD and Catalase mimetics in VSMC.

Serum-starved cells were loaded with 50 - 200 μM MnIIITM (A) or 50 - 200 μM EUK (B) for 30 min or 2 h. Phosphorylation of proteins was detected by western blot analysis after stimulation with 100 nM Ang II for 10 min, tubulin served as loading control. One blot for each kinase (pAkt, pp38, and pERK1/2) is shown; the top panel represents signals obtained after 30 min preincubation time, the bottom panel shows signals after 2 h pretreatment with MnIIITM and EUK. The experiment was performed one to three times.

Due to unconvincing effects of the other antioxidative compounds on Akt phosphorylation after Ang II stimulation we did not include additional experiments using EGF as activator of Akt phosphorylation.

3.5. Oxidation of Shp2 after Ang II and EGF treatment

The thiol groups of cysteins in protein tyrosine phosphatases (PTPs) are very important for their action and are also target of oxidation leading to inactivation of PTP. Shp2, a PTP, plays a pivotal role in Akt and ERK signaling, negatively influencing Akt and activating ERK. A recent publication indicated that Shp2 is redox-regulated by Ang II. Shp2 was shown to be activated by RV, possibly by interfering with Ang II- or EGF-mediated oxidation of Shp2. In order to test a redox-dependent effect of RV on Shp2 we applied a specific assay to detect oxidized PTPs using an antibody directed against the sulfonyl acid form of phosphatases.
Figure 24: Oxidation of Shp2 after Ang II stimulation

After incubation of quiescent cells with 100 nM Ang II and 3 mM H$_2$O$_2$ for indicated times an IP of Shp2 was performed. One half of the samples were treated with IAA to protect reduced (active) forms of Shp2, the other half was used without IAA (positive control). Protein levels were detected by western blot analysis, using an anti-oxidized PTP and Shp2 antibody. The graph represents the ratio of oxidized PTP in IAA treated cells and positive control cells (without IAA), related to the total Shp2 amount after IP (A). One representative blot out of three individual experiments is shown (B).

Cells were stimulated with 100 nM Ang II for different times, and Shp2 oxidation was measured with the oxidized PTP-assay as described in the part Materials and Methods. H$_2$O$_2$ was used as positive control. A ratio of the amount of oxidized PTP with IAA to protect reduced (active) forms of Shp2 and without IAA (positive control) was calculated, and related to the total Shp2 amount. Interestingly, Shp2 was already oxidized in untreated samples, indicating a high basal oxidation level of Shp2. As shown in the graph and also in the representative blots no further oxidation of Shp2 was detectable within 10 min treatment with Ang II. Even the positive control (3 mM H$_2$O$_2$, 10 min) did not lead to a higher oxidation percentage of oxidized Shp2 compared to the control (Fig. 24A-B). When applying the same assay in cells pretreated with RV (50 μM, 30 min) or 0.1 % DMSO and then stimulated with Ang II (100 nM, 10 min) or EGF (100 ng/ml, 5 min) no induction in oxidation could be seen after Ang II (Fig. 25A) or EGF (Fig. 25B) stimulation or RV pretreatment.
Figure 26: Non-redox active derivatives of RV

In contrast to RV, which possesses three phenolic groups the derivative Tri-O-methylresveratrol (TM-RV) has all three hydroxyl groups methylated (A), the derivative 3,5-Dihydroxy-4'-methylstilben (R1) is methylated at the 4'OH (B). Synthesized by the group of Prof. Erker (Department of Pharmaceutical Chemistry, Uni. of Vienna)

3.6 Effect of redox-inactive derivatives of RV on Akt activation

To further clarify the role of RV as a redox-active compound when inhibiting Akt phosphorylation in our cell system we applied two redox-inactive derivatives of RV, Tri-O-methylresveratrol (TM-RV), with all three hydroxyl groups methylated (Fig. 26A), and 3,5-Dihydroxy-4'-methylstilben (R1), methylated at the 4'OH (Fig. 26B). The phenol groups at the 3, 4' or 5-position, particularly the 4'OH, was shown to be important for the depletion of ROS by RV.27-29

Figure 25: Shp2 oxidation after RV pre-treatment and Ang II or EGF stimulation

Serum-starved cells were stimulated with 100 nM Ang II for 10 min or 100 ng/ml EGF for 5 min after preincubation with 50 μM RV for 30 min or 0.1 % DMSO. 3 mM H₂O₂ stimulation of the cells for 10 min served as positive control. Shp2 was immunoprecipitated, using IAA in one half of the samples and for positive control IAA in the other half. Protein levels were detected by western blot analysis, using an anti-oxidized PTP antibody and a Shp2 antibody. The experiment was carried out one to two times; one representative blot per stimulation is shown.
Neither of the two derivatives was found to significantly reduce $H_2O_2$ levels as RV did (Experiment performed by Julia Gesslbauer, diploma student; data not shown).

We therefore pretreated the cells with the derivatives TM-RV and R1 (50 μM, 30 min) and as positive control with RV (50 μM, 30 min) and then stimulated the cells with Ang II (Fig. 27A) or EGF (Fig. 27B) as previously described. TM-RV inhibited neither the Ang II nor EGF induced Akt phosphorylation. Interestingly, the derivative R1, which has been shown not to be redox active, reduced phosphorylation of Akt after Ang II treatment by over 50 %, to a level comparable to RV-mediated inhibition. EGF-induced phosphorylation of Akt was even inhibited equally well by R1 compared to the effect of RV. This data clearly shows that RV can inhibit Akt phosphorylation even without antioxidative properties in both Ang II and EGF stimulated VSMC. The 4’ phenolic group hereby seems not to play a pivotal role.

Figure 27: Non-redox active derivatives of RV can inhibit the phosphorylation of Akt after Ang II and EGF stimulation
Serum-deprived cells were pretreated with RV or RV derivatives TM-RV and R1 for 30 min, followed by stimulation with 100 nM Ang II for 10 min (A) or 100 ng/ml EGF for 5 min (B). Protein levels were detected by western blot, one representative blot per stimulation is shown. Graph represents mean densitometric signal of three independent experiments. Ang II or EGF stimulation is set to 100 % (**, p < 0.01 one-way ANOVA versus Ang II- or EGF-treatment).
This experiment was performed by Julia Gesslbauer (diploma student in our group).
4. Redox-unrelated mechanisms of RV influencing Shp2

4.1. Phosphorylation of Shp2

4.1.1. Shp2 phosphorylation at Tyr\textsuperscript{542} in response to Ang II or EGF stimulation

Beside redox regulation of Shp2 it was shown to be activated via phosphorylation of tyrosine-residues\textsuperscript{131, 140}. In a non-active Shp2 the N-terminal SH2 domain and the catalytic domain interact, rendering a low basal activity. Shp2 can be activated either by binding phosphorylated tyrosines of binding proteins, such as Gab1, with the two SH2 domains or by phosphorylation of the two tyrosine residues (Tyr\textsuperscript{542}, Tyr\textsuperscript{580}) at the C-terminus of Shp2.\textsuperscript{131, 140} An induction of phosphorylation of Shp2 after Ang II stimulation was reported recently\textsuperscript{149}, therefore we checked the phosphorylation status of Shp2 after Ang II and EGF treatment at Tyr\textsuperscript{542}.

Cells were stimulated with 100 nM Ang II for 10 min or 100 ng/ml EGF for 5 min after pretreating them with RV or vehicle control for 30 min. The western blot analysis using an antibody against phospho-Tyr\textsuperscript{542}-Shp2 revealed that after Ang II stimulation the phosphorylation of this tyrosine residue is induced significantly by more than 60 %. Pretreatment with RV even enhanced this phosphorylation for additional 20 %. Interestingly, upon EGF treatment or RV preincubation followed by EGF stimulation the

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**Figure 28: Difference in the phosphorylation of Shp2 upon Ang II or EGF treatment**

Shp2 phosphorylation at Tyr\textsuperscript{542} was detected in quiescent cells which were loaded with 50 μM RV or vehicle control for 30 min, then treated with 100 nM Ang II (10 min) or 100 ng/ml EGF (5 min). Following western blotting, the band intensities were densitometrically analyzed, the graph shows the mean of three independent experiments. One representative blot is shown for pShp2, total Shp2 and tubulin. Data were normalized using Ang II stimulation as 100 % (**, p < 0.01, ns, not significant; t-test).
Shp2 phosphorylation did not increase significantly (Fig. 28). According to these results we conclude that the phosphorylation of Shp2 is differently regulated and modulated by RV in Ang II and EGF signaling pathways.

### 4.1.2. Antioxidant treatment can not reduce the phosphorylation of Shp2

The Ang II stimulation in VSMC is leading to a transactivation of the EGF-R and Nox1 plays an important role for the signal transduction\(^{165}\). ROS production was already shown to induce the phosphorylation and activity of Shp2\(^{149}\). Due to this we examined whether the enhanced phosphorylation of Shp2 after Ang II stimulation is also redox regulated in our cell system. Cells were incubated with 50 μM RV, 0.1 % DMSO (30 min), 10 μM DPI (1 h) or 10 mM NAC (2 h) followed by stimulation with Ang II (100 nM, 10 min). We found that the phosphorylation of Shp2 was not reduced significantly by NAC or DPI preincubation followed by Ang II activation. Compared to RV treatment, NAC led to a statistically significant downregulation of Shp2 phosphorylation, suggesting that RV influences the phosphorylation of Shp2 not mainly via a redox-dependent mechanism (Fig. 29).

**Figure 29: Influence of antioxidants on Shp2 phosphorylation**

After pre-treatment of serum-starved cells with 50 μM RV or 0.1 % DMSO for 30 min, incubation with 10 μM DPI (1 h) or 10 mM NAC (2h), cells were stimulated with 100 nM Ang II for 10 min. Proteins were subjected to western blot analysis for pShp2 and tubulin (loading control). Graphs represent mean densitometric signals from four independent experiments. Ang II stimulation is set to 100 % (**, p < 0.01; *, p < 0.05; ns, not significant; t-test).
4.1.3. Phosphorylation of Shp2 in response to Ang II stimulation occurs mainly independent of the EGF-R

Prior to regulation of ERK1/2 signaling and PI3K activity after EGF stimulation Shp2 is recruited to the EGF-R. Elucidating the disparate regulation of the phosphorylation of Shp2 after Ang II and EGF stimulation (Fig. 29) strongly argues for an EGF-R and thus EGF-R transactivation-independent way of Shp2 phosphorylation upon Ang II stimulation.

To test this hypothesis cells were pretreated with an EGF-R-kinase inhibitor, AG1478 (250 nM, 30 min) and stimulated with Ang II (100 nM, 10 min). As shown in the graph the phosphorylation of Shp2 upon Ang II stimulation could not be abolished with AG1478 treatment (Fig. 30). This result suggests that, beside the EGF-R transactivation, Ang II acts on Shp2 via a different, parallel pathway.

![Phosphorylation of Shp2](image)

**Figure 30: EGFR-kinase activity is not important for the phosphorylation of Shp2 after Ang II stimulation**

Phosphorylation of Shp2 was detected by western blotting after incubation of quiescent VSMC with 250 nM AG1478 and 50 μM RV or vehicle control for 30 min, followed by stimulation with Ang II (100 nM, 10 min). Graphs represent the mean densitometric signal of two to four independent experiments. Data were normalized setting Ang II stimulation to 100 % (ns, not significant; one-way ANOVA versus Ang II-treatment).

4.2. Ang II-induced phosphorylation of Akt, p38 and ERK1/2 are partly independent of EGF-R kinase activity

To clarify whether the signal from the AT1R towards Akt, ERK1/2 and p38 phosphorylation require the transactivation of the EGF-R we activated cells with Ang II and blocked the EGF-R with the EGF-R-kinase inhibitor AG1478. VSMC were treated with AG1478 (250 M, 30 min) or 50 μM RV (30 min) and then stimulated with 100 nM Ang II for 10 min. Western blot analysis showed that Akt and p38 phosphorylation after Ang II stimulation was reduced to 50 % by AG1478 (Fig. 31A-B) and the phosphorylation of ERK was even blunted to 30 % in the presence of AG1478 (Fig. 31C). But still a phosphorylation of
all three kinases could be seen after Ang II stimulation. The effect of AG1478 was not comparable to the RV-mediated inhibition as this acts selectively on Akt phosphorylation but does not block ERK1/2 or p38 phosphorylation.

As control for the effectiveness of the inhibitor, cells were stimulated with 100 ng/ml EGF for 5 min after 30 min pretreatment with 250 nM AG1478 or 50 μM RV. Phosphorylation of Akt, p38 and ERK was detected by Western blot analysis using specific antibodies. As expected, AG1478 pretreatment led to a total inhibition of phosphorylation of the three kinases to basal level. RV only blocked the phosphorylation of Akt, but did not influence ERK or p38 activation significantly (Fig. 32A-C). These data support the idea that in VSMC 20 - 50 % of the Ang II-induced signal for Akt, p38 and ERK are transduced independently of the EGF-R. Furthermore, they show that the EGF-R kinase activity is absolutely required for EGF-induced Akt, p38 and ERK1/2 signaling in VSMC.
4.3. Recruitment of PI3K to Shp2

Shp2 was shown to regulate PI3K binding to Gab1, which is needed for activation of the Akt pathway in fibroblasts in response to EGF stimulation. Therefore we examined a possible interaction of Shp2 and PI3K in VSMC upon stimulation. To further elucidate the role of PI3K in our system we performed an IP of Shp2 after Ang II or EGF stimulation and RV pretreatment and analyzed the co-precipitation of p85, the regulatory subunit of PI3K, with a specific antibody. As Fig. 33 shows, no p85 recruitment to Shp2 could be detected after Ang II treatment. In contrast, EGF stimulation led to a significant increase of PI3K-Shp2 interaction, arguing for a co-localization in a “Shp2-PI3K-signaling complex” of both. This indicates that PI3K is differently regulated in Ang II and EGF signaling and might play a distinct role. RV pretreatment does not change the recruitment after Ang II- or EGF-mediated activation of the cells.
Figure 33: Differences in PI3K recruitment to Shp2 after Ang II and EGF stimulation

After pre-treatment with RV (50 μM, 30 min) or vehicle control quiescent cells were stimulated with 100 nM Ang II (10 min) or 100 ng/ml EGF (5 min). After lysis of the cells an IP of Shp2 was performed and proteins co-precipitated were detected by western blotting. A representative blot is shown for p85 and Shp2. Graph shows mean densitometric signal of three individual experiments, related to total Shp2 amount after IP. Ang II stimulation was set to 100 % (**, p < 0.01; *, p < 0.05; ns, not significant; t-test).

4.4. Shp2 knock down

As the previous data indicate a different role for Shp2 in the EGF and Ang II signaling we wanted to clarify its importance for the signaling and also examine whether it is the main target of RV. Therefore we treated the cells with 50 μM Shp2 siRNA for 72 h, added RV for 30 min and stimulated the cells with Ang II and EGF. Protein levels were analyzed by western blotting and densitometric analysis. The Shp2 levels in VSMC were reduced to 50 % compared to scrambled siRNA treated cells. Comparing the siRNA treated cells with the scrambled control cells neither the induction of Akt phosphorylation by Ang II or EGF nor the RV-mediated inhibition of phospho Akt was significantly influenced by the changed Shp2 protein level (Fig. 34A-B).
Figure 34: No influence of 50 % Shp2 knock down on Ang II- or EGF-stimulated Akt signaling and RV-mediated inhibition of phospho Akt

Serum-starved cells were treated with 50 μM siRNA for Shp2 or 50 μM scrambled control siRNA followed by incubation with RV or vehicle control for 30 min. Then cells were stimulated with 100 nM Ang II, 10 min (A) or 100 ng/ml EGF, 5 min (B). Protein levels were detected by performing western blotting and subsequently densitometric analysis of bands. A representative blot is shown for pAkt, total Shp2 and tubulin. Four independent experiments are summarized in the graphs, showing the mean band intensities. Data were normalized using Ang II or EGF stimulation of scrambled control as 100 % (**, p < 0.01; t-test).

5. Additional potential mechanisms of RV

5.1. Kinetics of RV-mediated inhibition of Ang II and EGF-induced phosphorylation of Akt

The inhibition of Akt phosphorylation by RV was always shown after a preincubation time of 30 min (Fig. 11). To shed more light on the mode of action of RV we tested whether RV can also inhibit Akt phosphorylation when applied after Ang II or EGF stimulation. Cells were stimulated with either 100 nM Ang II or 100 ng/ml EGF and RV was added up to 10 min after the stimulation (Fig. 35A-B). Surprisingly, addition of RV even 7 or 9 min after Ang II still led to a significant inhibition of the phosphorylation of Akt by ~40 %. Inhibition of Akt phosphorylation by RV was below 50 % when added up to 7 min after Ang II. Application of RV 5 to 7 min after Ang II stimulation resulted in higher inhibition rates, and when added within 2 min after Ang II phospho-Akt levels were comparable to prestimulation with RV.

Results
Stimulating cells with EGF, RV inhibited phosphorylation of Akt when added within 4 min after EGF-application. Addition of RV up to 3 min after stimulation with EGF blocked the Akt phosphorylation to nearly the same extent like RV preincubation.

Figure 35: RV treatment after Ang II- and EGF stimulation inhibits phosphorylation of Akt

Serum-starved cells stimulated with 100 nM Ang II (A) for 10 min or 100 ng/ml of EGF for 5 min (B) were treated with 50 μM RV for indicated the times. Lysates were subjected to western blot analysis using antibodies to pAkt and tubulin (loading control). 0.1 % DMSO was used as vehicle control. One representative blot is shown; graphs represent signal intensity detected by densitometric analysis of three to four immunoblots. Ang II or EGF stimulation, respectively, was set to 100 % (**, p < 0.01; ns, not significant; one-way ANOVA versus Ang II-or EGF-treatment).

5.2. PI3K as target

To get more ideas about the mode of action of resveratrol we examined different stimuli next to Ang II and EGF which are all activating Akt via PI3K. PI3K is an important modulator downstream of the EGF-R, binding to Gab1 and leading to Akt phosphorylation. RV was previously shown to inhibit PI3K activity, which could explain the inhibition of phosphorylation of Akt after stimulation with Ang II and EGF.

We therefore used insulin, a potent stimulus of PI3K, and investigated whether RV can also inhibit insulin-mediated Akt phosphorylation. A 10 or 30 min incubation of VSMC with 100 nM insulin led to a 3-fold increase in the phosphorylation of Akt, comparable to Ang II stimulation (Fig. 36). RV completely blocked this phosphorylation reducing phospho-Akt-levels even below basal levels, similar to the effect on Ang II stimulated cells.
Figure 36: Insulin induced phosphorylation of Akt in VSMC
Quiescent cells were stimulated with 100 nM Insulin for 10 min or 30 min. Protein detection was performed by immunoblotting and mean intensity measured by densitometric analysis of three individual experiments is shown for pAkt. One representative blots is shown. Insulin stimulation was set to 100 % (**, p < 0.01; ns, not significant; t-test).

We next compared the inhibition of Akt phosphorylation to the effect of Wortmannin (WM), a potent PI3K inhibitor. Cells were stimulated with 100 nM Insulin for 10 min after a 30 min preincubation with 50 μM RV or 50 nM WM. The detection of the protein levels with western blotting revealed that RV can block the insulin induced phosphorylation of Akt nearly as powerful as WM (Fig. 37). Since RV turned out to inhibit phosphorylation of Akt upon various stimuli (Ang II, EGF, Insulin) and PI3K is upstream of pAkt, PI3K may constitute a target of RV in VSMC.

Figure 37: RV and WM block phosphorylation of Akt in VSMC
Serum-starved VSMC were stimulated with 100 nM Insulin for 10 min after preincubation with 50 μM RV, 0.1 % DMSO or 50 nM WM for 30 min. Western blotting was used for protein detection and mean of densitometric analysis is shown. One out of three representative blots is shown for pAkt and tubulin. Data were normalized using Insulin stimulation as 100 % (**, p < 0.01; one-way ANOVA versus Insulin-treatment).
5.3. Sirtuins as target of RV

An *in vitro* screening identified RV as a potent inducer of the of huSIRT1 deacetylase activity. Many different effects of RV were found to be due to activation of sirtuins, therefore we applied Sirtinol, a potent SIRT1 inhibitor effective at low concentrations to test whether the RV effect of Ang II- and EGF-mediated signaling is also SIRT1-dependent. Cells were treated with 10 μM Sirtinol for 15 min or RV (50 μM, 30 min), followed by stimulation of the cells with Ang II (100 nM, 10 min) or EGF (100 ng/ml, 5 min). Pretreatment of the cells with Sirtinol did not abolish inhibitory effect of RV on the phosphorylation of Akt, while preincubation of the cells with Sirtinol alone also did not influence the Ang II- or EGF-induced phosphorylation of Akt (Fig. 38A-B). Referring to these results we exclude an involvement of sirtuins in the repression of Akt phosphorylation by RV in the VSMC.

**Figure 38: Sirtinol does not influence the RV-mediated inhibition of phosphorylation of Akt in VSMC**

Sirtinol (10 μM, 15 min) pretreated serum-deprived cells were incubated with RV (50 μM) for 30 min before stimulation or stimulated directly with Ang II (100 nM, 10 min) (A) or EGF (100 ng/ml, 5 min) (B). 0.1 % DMSO for 30 min was used as vehicle control. Cell lysates were subjected to immunoblotting using antibodies to pAkt and tubulin. Protein detection was performed by measuring densitometric analysis. One representative blot is shown. Graphs show mean of densitometric analysis of three independent experiments. Data were normalized using Ang II or EGF-stimulation as 100 % (**, p < 0.01; *, p < 0.05; t-test).
E  Discussion

It has previously been shown by our group that RV can inhibit Ang II-induced hypertrophy in VSMC by interfering with the Akt/PI3K pathway. Further it was suggested that RV blocks Akt activation after Ang II- or EGF stimulation by activating Shp2, thus interfering with the interaction of Gab1 and PI3K that is necessary for the signaling to Akt42-44. In this study we tested whether this effect of RV is due to its antioxidative properties by influencing the redox-sensitive phosphatase Shp2 and/or NADPH oxidases downstream of the EGF-R as previous data showed that the EGF-R transactivation is not influenced by RV. The study showed that although RV could inhibit intracellular and extracellular ROS, the phosphorylation of Akt is redox-independent downstream of the EGF-R in VSMC. Nox1 was shown to be responsible for the Akt, p38 and ERK1/2 phosphorylation after Ang II stimulation which is in accordance with the literature. EGF- induced Akt, p38 and ERK1/2 phosphorylation in contrast was Nox1 independent. We also found that Nox4 is not involved in Ang II- or EGF-induced Akt and MAPK (p38/ERK1/2) phosphorylation. Using redox inactive derivatives of RV in this setting underlined that antioxidative properties of RV are not necessary for the inhibition of Akt phosphorylation. In addition we could not find evidence for a redox regulation of Shp2 using oxPTP antibodies, but detected differences in the phosphorylation of Shp2 and in PI3K recruitment to Shp2 upon stimulation of cells with Ang II or EGF. Our findings therefore have led to a more complete understanding and of the mode of action of RV against VSMC hypertrophy by excluding an antioxidative effect of RV.

1. Antioxidative effect of Resveratrol

RV, like other polyphenols, is known for its antioxidative effect200 and it was suggested to inhibit Ang II and EGF-mediated Akt phosphorylation possibly by interfering with ROS production downstream of the EGF-R . In this study we could show for the first time that RV also acts as an antioxidant in rat VSMC. RV was able to inhibit Ang II-induced ROS levels in our cell system, and led to reduced basal intracellular ROS levels, which were comparable to those upon administration of the flavoprotein inhibitor DPI. Similar data regarding inhibition of ROS production by RV was obtained in cardiomyocytes45, in human aortic VSMC (inhibition of PDGF-induced ROS)223, and in bovine smooth muscles cells (inhibition of oxLDL induced ROS)224 as well as in various other cells systems9,225. As EGF-R
phosphorylation was shown to be susceptible for modulation by ROS\textsuperscript{226} and the EGF-R was found to also generate H\textsubscript{2}O\textsubscript{2} after stimulation of A431 human epidermoid carcinoma cells with EGF\textsuperscript{227}, we looked if the H\textsubscript{2}O\textsubscript{2} production in our cell system is triggered by EGF-R activation. However, we were not able to detect any intracellular ROS-increase after EGF stimulation, which could be due to a high basal cellular ROS level already present in unstimulated VSMC. Still, RV was able to reduce ROS below levels found in vehicle control treated cells, indicating an antioxidative role of RV. Further, extracellular ROS, which were enhanced by Ang II or EGF stimulation, were also brought down below basal levels by RV. The increase of extracellular ROS was in accordance with literature where extracellular H\textsubscript{2}O\textsubscript{2} production is due to an activated EGF-R.\textsuperscript{203} These antioxidative properties of RV are thought to include several mechanisms. RV was shown to directly scavenge ROS\textsuperscript{21}, \textsuperscript{228}, and also to upregulate the expression of endogenous antioxidants such as SOD and catalase in SMC, with measurable effects 24 h after RV-addition\textsuperscript{25}. In our experimental setting, RV already blunted ROS after a 30 min preincubation. Therefore, we can exclude the latter in our system, leaving direct ROS scavenging as a potential explanation for the strong effect of RV on intra- and extracellular ROS. Another possibility would be that RV acts on flavoproteins (e.g. Noxes) like DPI which was shown to inhibit phosphorylation of Akt in VSMC\textsuperscript{211}.

1.1. Role of Noxes in Ang II- and EGF-induced signaling

Noxes, especially Nox1, seem to be important for the regulation of Ang II induced signaling as they are necessary for transactivation of the EGF-R.\textsuperscript{229} Further, Nox4 was found colocalized with the activated EGF-R, indicating a role of both Noxes in Ang II and EGF signaling.\textsuperscript{181, 191} In cardiomyocytes Ang II was shown to induce O\textsubscript{2}\textsuperscript{•−} in a Nox2 and Rac1 dependent way leading to Akt activation and hypertrophy.\textsuperscript{230} RV has already been reported as suppressor of Ang II-induced Nox activity in endothelial cells\textsuperscript{48} and it was shown to inhibit LPS-induced Nox1 expression in macrophages leading to inhibition of foam cell formation\textsuperscript{231}. This all implicates an impact of RV in Nox regulation. In addition, a role of Nox 4 downstream of the EGF-R was proposed, as other groups found a Nox4-based but Rac-dependent ROS production in rat mesangial cells that was shown to be important for Ang II induced protein synthesis via Akt.\textsuperscript{189} Moreover, it was also shown before that the EGF-R is able to produce H\textsubscript{2}O\textsubscript{2} upon stimulation\textsuperscript{203}, to co-localize with Nox4 in VSMC when activated\textsuperscript{181}, and to inactivate PTP1B that is localized at the EGF-R in HAECs\textsuperscript{232}.  

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Discussion
A lack of specific antibodies against Noxes made it impossible to detect Nox protein levels after siRNA treatment of the cells. Therefore mRNA levels were analyzed to test for sufficient Nox4 reduction. Stimulation of cells, that have been depleted of Nox4 by a specific siRNA, with Ang II or EGF, did not lead to a difference in the phosphorylation of Akt, p38 or ERK when compared to nonsense RNA-treated and stimulated cells. Also no changes in the RV-caused inhibition of phosphorylated Akt were seen upon treatment with Nox4 siRNA. Therefore we could rule out that Nox4 plays a pivotal role in Ang II or EGF signaling and that RV mediates its effect via Nox4 inhibition in VSMC.

The role of Nox1 in Ang II signaling has been better characterized, suggesting ROS produced by Nox1 to be important for the EGF-R transactivation, and also suggesting a clear dependence of Akt and p38 phosphorylation on Nox1 upon Ang II stimulation. In addition, data in HEK cells indicate a role of Nox1 in Ang II-induced ROS production, and reports showed that Rac1 is needed for the stimulation of NADPH oxidase activity by Ang II. Other groups also showed, that the AT1R and Nox1 are co-localized in caveolae, indicating an interaction during Ang II signaling. Using a Nox1 blocking peptide that inhibited the assembly of Nox1 and therefore its function, we found that Ang II-induced phosphorylation of Akt and p38 was inhibited significantly. This underlines the importance of Nox1 in Ang II signaling and shows a clear dependence of the Ang II signaling pathway on Nox1. In contrast, EGF-mediated phosphorylation of Akt and MAPK was not significantly altered in cells with impaired Nox1-assembly. Another publication found Nox1 as a main source for Akt activation upon Ang II stimulation only in VSMC of spontaneously hypertensive rats, not in VSMC of control animals. A possible explanation might be that our cultivated VSMC display a phenotype differing from their smooth muscle cells. In addition a Nox1 regulation of Ang II signaling in VSMC was also found by other groups supporting our results. To sum up our results we exclude a redox-dependent mechanism downstream of the EGF-R but point out a pivotal role of Nox1 in the Ang II signaling, while no influence of Nox4 was found. Further, RV was not found to inhibit Nox1 or Nox4.

Application of AEBSF, a Nox inhibitor, to the cells led to some reduction of ROS produced after Ang II stimulation, similar to data described in the literature whereas even an increase of Akt phosphorylation was detected in our cells. Therefore we concluded that Nox inhibition in VSMC by AEBSF in the concentrations tested (50 μM - 1 mM) was not potent enough to inhibit Akt phosphorylation, although the ROS level was decreased when pretreating the cells with 100 μM AEBSF.
Downstream of the EGF-R, PI3K was suggested as a main player involved in ROS production in VSMC\textsuperscript{164}. Transactivation and phosphorylation of the EGF-R after Ang II stimulation was already shown to be redox-sensitive, whereas no ROS is needed for a direct activation of the EGF-R with EGF\textsuperscript{229}. However, redox-dependent signaling steps downstream of the EGF-R were suggested in VSMC\textsuperscript{164}. In agreement with these findings we found an abrogation of Akt and p38 phosphorylation upon Ang II stimulation following pretreatment with DPI and NAC. Concerning ERK1/2 phosphorylation, we only detected an inhibition by NAC, confirming experiments by Frank et al\textsuperscript{70}, while other groups also reported an inhibition of Ang II-mediated ERK1/2 phosphorylation by DPI\textsuperscript{71}. Reason for this discrepancy is unknown, although differences in cellular systems used might be one explanation. Interestingly, when stimulating cells with EGF, we could not detect any influence of DPI and NAC on Akt phosphorylation, suggesting that despite the extracellular ROS production after EGFR transactivation\textsuperscript{164} ROS are dispensable for this signaling step. In summary, our data indicate that the transactivation of the EGF-R by Ang II stimulation is ROS dependent, but we could not find evidence for redox sensitive downstream signals of the EGF-R leading to Akt phosphorylation. Moreover, we found differences in the phosphorylation of MAP-kinases upon Ang II-stimulation when pretreating with DPI and NAC antioxidants compared to preincubation with RV. RV interferes specifically only with Akt phosphorylation, whereas DPI and NAC also diminished the phosphorylation of p38. In agreement with previous data of our group these findings strongly indicate that also other than antioxidant properties of RV play a role in the inhibition of Akt-phosphorylation. Previous findings showed that the transactivation of the EGF-R by Ang II was shown to be dependent on Noxes\textsuperscript{229, 237}. RV, however, did not interfere with this transactivation, in contrast to NAC\textsuperscript{44}. This is further supporting the assumption that RV is not mainly acting as antioxidant when interfering with Akt phosphorylation.

By using additional antioxidants and redox-active compounds other than DPI, which is a rather unspecific flavoprotein inhibitor, and NAC, a general antioxidant\textsuperscript{238}, we wanted to further clarify the role of ROS in Ang II and EGF-mediated signaling in VSMC. In addition the hydroxyl radical scavengers DMTU and MPG were also tested concerning their effect on Akt phosphorylation. DMTU was shown to inhibit E-Hb-induced hydroxyl radicals in human aortic SMC\textsuperscript{239}. In human VSMC also a reduction in intracellular hydroxyl radicals after oxLDL incubation was shown when applying 10 mM DMTU\textsuperscript{240} which proofed that DMTU is a hydroxyl radical scavengers in SMC. By preincubation of the cells with 50 μM - 10 mM DMTU, no influence in Akt phosphorylation after Ang II stimulation was detectable. Differences between our results and the literature can be due to distinct experimental setups and cell types as both DMTU effects of other groups were found in human but not rat VSMC. An effectiveness of MPG was shown in vivo in myocardial ischemia-reperfusion (IR) injury models by influencing positively post ischemic myocardial dysfunction when
acting as hydroxyl scavenger and preventing \( \text{H}_2\text{O}_2 \)-induced left ventricle dysfunction\(^{215}\). Also in TNF-\( \alpha \) treated myoblasts MPG at a concentration of 10 \( \mu \text{M} \) was shown to inhibit ROS production\(^{241}\) and the ET-1-induced positive inotropic effect in rat myocytes was inhibited by 2 mM MPG. As MPG was shown to have antioxidative effects in various cell systems we tested 50 - 300 \( \mu \text{M} \) MPG on our cells and found that it did not significantly influence Akt phosphorylation in the tested concentrations. As MPG preincubation in some experiments showed a slight inhibition of Akt phosphorylation, we cannot completely rule out an effect of MPG, but this would have to be investigated further with varying concentrations and preincubation times.

We next investigated, whether the SOD and catalase mimetics EUK 134 and MnIIITM in various concentrations could mimic the RV effect in our cells. EUK134 was shown to act in myocytes on LPS-induced ROS\(^{216}\) and decrease IL-\( \beta \)2 induced MMP-2 levels in endothelial cells\(^{242}\), MnIIITM was shown to similarly reduce IL-\( \beta \)2 induced MMP-2 levels in endothelial cells\(^{242}\). In our system, however, EUK34 and MnIIITM did not affect Akt-phosphorylation after Ang II pretreatment.

As none of the tested inhibitors was able to mimic the RV effect by inhibition of Akt phosphorylation, we concluded that RV is not mainly acting as hydroxyl radical scavenger or SOD or catalase mimetic and we also found that hydroxyl radicals are not important in the Ang II signaling.

1.2. Structure-activity relationship of RV and its derivates concerning Akt inhibition

To get a better understanding about the importance of the antioxidative properties of RV when interfering with Ang II and EGF stimulation, RV derivatives, that are methylated on one or more –OH groups (synthesized by the group of Prof. Erker) and therefore were shown to be non-antioxidative\(^{27-29}\), were applied to VSMC. The derivates R1 and TM-RV were not as potent in reducing \( \text{H}_2\text{O}_2 \) level as RV (experiment performed by Julia Gesslbauer, diploma student; data not shown). TM-RV, methylated at all hydroxyl groups, was not able to inhibit the phosphorylation of Akt, whereas the R1 derivate, also considerably less redox active because of the methylation on the 4´OH group\(^{27}\), could inhibit phosphorylation as potent as RV. This is in accordance with our previous findings showing that the antioxidative property of RV is not important for the effect on Akt phosphorylation. It also indicates a pivotal role of the 3´and 5´ OH groups which are not methylated in this derivative for the RV-mediated inhibition of Akt phosphorylation.

Discussion
Studies suggest that methylation protects dietary flavonoids and possibly also polyphenols from a rapid hepatic metabolism as the free hydroxyl groups of most polyphenols favour a very rapid conjugation by glucuronidation and sulfation\textsuperscript{243, 244}. Thus methylation could increase metabolic stability and therefore the human oral bioavailability, which would be important when using RV for oral formulations.\textsuperscript{245} Still, our study showed that methylation of the hydroxyl groups can interfere with the biological effects of RV, and therefore consumers should act with caution when using a metabolically more stable but methylated derivative of RV.

2. Shp2 and RV action

2.1. Shp2 and ROS

A previous publication indicated that Shp2 can influence ERK1/2 and Akt signaling downstream of the EGF-R.\textsuperscript{220} By dephosphorylation of the PI3K binding site of Gab1 Shp2 can inhibit the Akt phosphorylation in mouse fibroblasts.\textsuperscript{154} Also a direct activation of Shp2 by RV was shown\textsuperscript{44}, leading to the assumption that Shp2 is a direct target of RV in VSMC when interfering with Akt phosphorylation. One possible mode for the activation of Shp2 by RV would be via keeping it in a reduced, active status by avoiding oxidation of Shp2 due to Ang II- or EGF-triggered ROS production. During our study we found no increase in the oxidation of Shp2 upon Ang II or EGF stimulation, although such a modification of Shp2 was shown in cardiac fibroblasts stimulated with Endothelin-1 (ET-1), that similarly utilizes signal transduction via the EGF-R.\textsuperscript{246} Interestingly, we found already oxidized Shp2 in unstimulated cells, which is in agreement with the high basal ROS levels detected in our cells. In the literature, contradictory data exists about the increase in Shp2-oxidation upon Ang II stimulation: A recent publication showed no oxidation of Shp2 after Ang II stimulation with an in-gel–assay, but could on the other hand measure an increase of oxidized Shp2 with an antibody-based assay, that was also used in this thesis.\textsuperscript{149} Additionally, another publication suggested that redox regulation of Shp2 strongly depends on the cell type and pointed out that the usage of antibodies against oxidized phosphatases \textit{in vitro} might not be sensitive enough to detect intracellular phosphatases.\textsuperscript{148} Therefore, the interpretation of our data collected with this antibody-assay is difficult, and no conclusion on a redox regulation of Shp2 after Ang II stimulation could be drawn. Moreover, it was recently shown that the stably oxidized form of Shp2 consists of a backdoor-backdoor disulfide bridge while the catalytic cysteine remains in a reduced state, which makes it problematic to detect oxidized...
Shp2 with techniques based on trapping of sulphenic acids or modified cysteins at the active site.\textsuperscript{135}

2.2. Shp2 phosphorylation in Ang II and EGF signaling

Similar to oxidization, phosphorylation of Shp2 is a posttranslational way of regulating Shp2 activity. As RV was shown to activate Shp2 activity\textsuperscript{44}, it was interesting to investigate, whether this is achieved by phosphorylation. When testing phosphorylation of Shp2 after Ang II or EGF stimulation we found a different pattern. Ang II induced a strong hyperphosphorylation of Shp2, whereas only a slight alteration was detectable after EGF treatment, confirming data from the literature\textsuperscript{145, 149}. Addition of RV had no significant effect on Shp2 phosphorylation; only a slight increase of Ang II-mediated Shp2 phosphorylation could be seen. In contrast, Ang II was shown to reduce phosphorylation of Shp2 in endothelial cells\textsuperscript{156}, indicating different effects in different cell types. Shp2 was further shown to attach to unstimulated AT1R, influencing Ang II-induced JNK/STAT signaling in fibroblasts\textsuperscript{247}, which could be an explanation for the different effects of Ang II and EGF in our cells.

Antioxidant treatment of cells showed that NAC was not able to inhibit Ang II-induced phosphorylation of Shp2, indicating no dependence of Shp2-phosphorylation on ROS. This is not in agreement with a report providing evidence that ROS (originated from Nox1) is important for Shp2 phosphorylation.\textsuperscript{149} For a final clarification also the influence of Noxes on Shp2 phosphorylation has to be tested. But as RV is not causing the same effect on Shp2 phosphorylation compared to antioxidants, it again excludes a major role of RV as redox-active compound in the signaling in VSMC.

The role of Shp2 for signaling processes, however, is controversially discussed in the literature. Beside its regulatory role of enhancing ERK1/2 signaling\textsuperscript{220} and the inhibition of p85 binding to the EGF-R via Gab1\textsuperscript{154}, other experiments using mouse embryonic fibroblast cells with a deletion at exon 3 (Shp2\textsuperscript{Ex3/-}) - a viable Shp2 knock out model - showed an influence of Shp2 on MAPK dependent on the stimulus. In that model PDGF led to a hyperphosphorylation of MAPK, while FGF was not able to induce an activation of MAPK when compared to wild-type cells.\textsuperscript{248} In VSMC, a different publication showed that Shp2 is activated and phosphorylated by Ang II and then inhibits MAPK activation by interfering with c-Src.\textsuperscript{249} Also a positive effect of Shp2 on Akt/PI3K after stimulation of fibroblasts with interleukin (IL)-3 is reported\textsuperscript{250}, as well as an activation of Shp2 during IGF-1 induced Akt activation.\textsuperscript{251} In addition, in cancer cells, but also in Shp2\textsuperscript{Ex3/-} fibroblasts, Shp2 was shown to activate Akt in dependence of EGF and PDGF.\textsuperscript{252} These publications lead to the
conclusion that Shp2 is a widely distributed phosphatase that can modulate MAPK and Akt activation differently, depending on the nature of the stimulus and the cell line used. Our data also indicate a different role of Shp2 in Ang II and EGF signaling. Therefore some general experiment concerning the role of Shp2 in the Ang II and EGF signaling in VSMC are needed to further define Shp2-dependent mechanism in this cell type.

2.3. Shp2 and PI3K

We showed that Ang II stimulation increases Shp2 phosphorylation, whereas EGF treatment had no effect on Shp2 phosphorylation. In addition, we found a EGF-induced p85 (PI3K subunit) recruitment to Shp2 (as obvious in co-immunoprecipitation) but no interaction after Ang II stimulation. Combining our findings on Shp2 phosphorylation with these data we suggest that Ang II stimulation leads to the phosphorylation and activation of Shp2 which in turn dephosphorylates the binding site for PI3K and inhibits PI3K recruitment to the Shp2 containing “signaling complex”. After EGF stimulation, however, Shp2 is not phosphorylated significantly and therefore PI3K can be recruited to Shp2, or the “signaling complex”, leading to a much stronger Akt signaling compared to the stimulation with Ang II. This hypothesis is supported by the data that Shp2 can dephosphorylate the binding site of p85 subunit of PI3K on Gab1. As RV did not seem to have any significant impact on Shp2 phosphorylation, a regulation of Shp2 phosphorylation is not an explanation for RV action.

A possible reason for the differences in Ang II and EGF signaling was shown by studies in different cell lines including cancer cells, Vero and HEK cells, stating that the PI3K activity is only important for Gab1- and Shp2-mediated ERK1/2 activation upon weak EGF signaling. When signal strength increases, the cells rather act via Grb2-mediated binding of Gab1 and Shp2, independent of PI3K. Experiments in our cells with WM, a PI3K inhibitor, in combination with EGF and Ang II stimulation both showed a clear dependence of Akt phosphorylation on PI3K, whereas ERK1/2 was not influenced by this inhibition (data not shown). Our data suggest that for Akt activation in our cells, PI3K is important, albeit the specific regulation might differ between the two stimuli. Still, as clear PI3K activity data in the presence of RV are missing, a mechanism of RV targeting PI3K cannot be ruled out yet.
Finally, Shp2 knock down indicates a marginal role of Shp2 on the RV effect, as knock down of Shp2 by 50 % did not influence the Akt-phosphorylation and RV effect significantly. However, because of the 50 % reduction, there still might remain enough Shp2 in the cells to mediate Akt phosphorylation and RV effect. Anyway we could not find any mechanism how RV is activating Shp2 and therefore inhibiting Akt phosphorylation after Ang II or EGF activation.

3. Ang II and EGF signaling

The transactivation of EGF-R by Ang II is triggered by the Ang II-induced cleavage of HB-EGF, that binds to the EGF-R72, activating a signaling cascade downstream of the receptor. Using AG1478, an EGF-R kinase inhibitor, we found no difference in the Ang II-mediated Shp2 phosphorylation. Referring to these data we showed that of Shp2 phosphorylation occurs upstream of the EGF-R, maybe via a regulation by the AT1R in VSMC. In addition, experiments with the EGF-R kinase inhibitor showed that only 50 % of the Akt phosphorylation in response to Ang II is regulated via the EGF-R. p38 activation seems to be controlled by the EGF-R at about 60 % and ERK1/2 phosphorylation at even 70 % in our cell system. The latter result is in contrast to other groups, that showed that ERK1/2 stimulation by Ang II-mediated signaling is completely independent of EGF-R transactivation in HEK cells254 and also in preglomerular SMC255, but that it seems to be dependent on c-src activation in VSMC256. In contrast other groups showed a total dependence of ERK1/2 phosphorylation88 and Akt phosphorylation257 after Ang II stimulation in rat VSMC on the EGF-R. Our data indicated a difference in Ang II and EGF induced signaling concerning the regulation of these kinases. When taking into account the literature a strong dependence on the cell type, stimulus and experiment setting is likely. Therefore additional experiments to clarify the signaling of Ang II in contrast to EGF downstream of the EGF-R in our cell system are needed.
4. **Other possible mechanisms of RV**

RV is a known activator of Sirtuins, linking it to increased life-span which led to its reputation as an anti-aging compound. We tested whether sirtuin plays a role for RV-mediated inhibition of Akt phosphorylation by using sirtinol, a sirtuin inhibitor. Similar to data obtained in mesengial cells where a sirtuin-independent inhibition of Akt after PDGF stimulation was found\(^\text{258}\), we were not able to detected a dependence of the RV effect on sirtuins in our cells. Therefore we could rule out a role of Sirtuin as modulator of the Akt phosphorylation, and consequently as a RV target, in our cell system.

RV is a phytoestrogen\(^\text{245}\) and was reported to selectively bind to $\alpha V\beta 3$ integrins on the cell surface\(^\text{259}\). A strong dependence of the RV effect on $\alpha$-estrogen receptor and integrins $\alpha V\beta 3$ in EGF- signaling was, however, excluded by our group. Other data showed that Shp2 slightly reverses EGF-induced FAK phosphorylation, and that this effect was overcome by RV (Dissertation Mario Kumerz, Univ. of Vienna, 2009). Together with minor changes after RV pretreatment on Shp2 phosphorylation a synergistic effect of RV on several molecules including Shp2 and FAK can not be ruled out at this moment and warrants further investigations.

In summary, we have investigated a possible mode of action of RV when inhibiting Ang II- or EGF-induced Akt phosphorylation after stimulation in VSMC. By acting as antioxidant RV was believed to inhibit Shp2 oxidation and therefore restore its activity leading to an inhibition of Akt phosphorylation. We could show that indeed RV is able to inhibit intra- and extracellular ROS in VSMC, but unexpectedly it did not act as antioxidant when inhibiting Akt phosphorylation. Further we identify Nox1 as major ROS source after Ang II but not EGF stimulation and therefore showed evidence that the signaling downstream of the EGF-R seems to be redox independent. In addition we rule out a role for Nox4 in these signaling pathways. We could not find any mechanism of RV-mediated Shp2 activation, but we detected differences in the phosphorylation of Shp2 and in PI3K recruitment to Shp2 upon stimulation of cells with Ang II or EGF. This work shows that RV is not just acting as an antioxidant in cells but that it has a more specific mode of action and therefore additional studies are needed to identify this very potent and selective effect of RV in VSMC.
REFERENCES
References


References


G Appendix

1. Abbreviations

A
AEBSF  4-(2-Aminoethyl)-benzenesulfonyl Fluoride
Ang II  Angiotensin II
ANOVA  Analysis of variance between groups
Akt  Protein kinase B
APS  Ammonium persulphate
AT1R  Angiotensin receptor 1

B
BSA  Bovine serum albumin

C
cDNA  Complementary DNA
CS  Calf serum

D
DCF  2’,7’-dichlorofluorescein
DMEM  Dulbecco’s modified Eagle’s medium
DMSO  Dimethyl sulfoxide
DMTU  Dimethyl thiourea
DNA  Desoxyribonucleic acid
dNTP  Deoxyribonucleotide triphosphate
ddH₂O  Double distilled water
DPI  Diphenyleneiodonium
DTT  Dithiothreitol

E
EC  Endothelial cells
ECL  Enhanced chemiluminescence
EDTA  Ethylenediamintetraacetic acid
EGF  Epidermal growth factor
EGF-R  Epidermal growth factor receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box-O transcription factors</td>
</tr>
<tr>
<td>Gab</td>
<td>Grb-2-associated binder (Gab)</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>Grb</td>
<td>Growth factor receptor binding protein</td>
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<td>GSK</td>
<td>Glycogen synthase kinase 3</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<td>H$_2$DCF</td>
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<td>H$_2$DCF-DA</td>
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<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulphonic acid)</td>
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<tr>
<td>IAA</td>
<td>Iodoacetic acid</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDA</td>
<td>kilo Dalton</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LMW-PTP</td>
<td>Low molecular weight protein-tyrosine phosphatase</td>
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<tr>
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<td>Mitogen activated protein kinase</td>
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<tr>
<td>Mc</td>
<td>Monoclonal</td>
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<tr>
<td>MnIIITM</td>
<td>Mn(III)terakis(1- methyl-4-pyridyl) porphyrin pentachloride</td>
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MPG  N-(2-mercaptpropionyl)-glycine
mRNA  Messenger RNA

N
NAC  N-acetyl-cysteine
NADPH  Nicotinamide adenine dinucleotide phosphate
NF-κB  Nuclear factor-κB
Nox  NADPH-oxidase

P
PAA  Polyacrylamide
PBS  Phosphate buffered saline
Pc  Polyclonal
PCR  Polymerase chain reaction
PDK1/2  Phosphoinositide-dependent protein kinase 1/2
PH domain  Pleckstrin homology domain
PI3K  Phosphatidylinositol 3-kinase
PIP2  Phophatidylinositol-4,5-bisphosphate
PIP3  Phophatidylinositol-3,4,5-trisphosphate
PKA  Protein kinase A
PKC  Protein kinase C
PKG  Protein kinase G
PMSF  Phenylmethylsulphonylfluoride
PTEN  Phosphatase and tensin homologue deleted on chromosome ten
PTP  Protein tyrosine phosphatase
PVDF  Polyvinylidenedifluoride

Q
qPCR  Quantitative real-time PCR

R
RNA  Ribonucleic acid
RNAi  RNA interference
ROS  Reactive oxygen species
RT  Reverse transcription
RTK  Receptor tyrosine kinase
RT-PCR  Real time PCR
RV  Resveratrol
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<td>Sodim dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>Src-homology 2 domain</td>
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<td><strong>SHIP</strong></td>
<td>SH2-containing inositol 5-phosphatase</td>
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<td><strong>Shp2</strong></td>
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<td><strong>SIRT1</strong></td>
<td>Sirtuin 1</td>
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<td><strong>SOD</strong></td>
<td>Superoxide dismutase</td>
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<td><strong>SOS</strong></td>
<td>Son of sevenless</td>
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<td><strong>Thr</strong></td>
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<tr>
<th><strong>V</strong></th>
<th><strong>VSMC</strong></th>
<th>Vascular smooth muscle cells</th>
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2. Publications


3. Poster Presentations


**Cornelia E. Schreiner**, Mario Kumerz, Elke H. Heiss, Verena M. Dirsch. The role of Shp2 and ROS in resveratrol-mediated inhibition of Akt phosphorylation in vascular smooth muscle cells. 5th International EDHF Symposium, Tampere (Finland), 24 - 27 June 2008

Mario Kumerz, **Cornelia E. Schreiner**, Elke H. Heiss, Verena M. Dirsch. Resveratrol inhibits EGF-induced Aktphosphorylation in vascular smooth muscle cells: role of integrin αVβ3, focal adhesion kinase and Shp-2. 5th International EDHF Symposium, Tampere (Finland), 24 - 27 June 2008
4. Short lecture

**Cornelia E. Schreiner**, Atanas G. Atanasov, Elke H. Heiss, Verena M. Dirsch. Role of Noxes and ROS in Resveratrol-mediated inhibition of Akt phosphorylation in Ang II or EGF-activated vascular smooth muscle cells. 34th FEBS Congress Life’s Molecular Interactions, Prague (Czech Republic), 4 - 9 July 2009 (*invited lecture*)
5. Curriculum Vitae

**Personal information**

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**Education**

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<td>10/1999 – 07/2005</td>
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**International stays**

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### Teaching experience

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### Other activities

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6. Acknowledgements / Danksagung

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