universität wien

DISSERTATION

Titel der Dissertation

Mechanistic studies on resveratrol and its influence on angiotensin II- and epidermal growth factor-induced signaling pathways in vascular smooth muscle cells

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

Verfasserin / Verfasser:	Cornelia Schreiner
Matrikel-Nummer:	9808547
Dissertationsgebiet (lt. Studienblatt):	Genetik - Mikrobiologie
Betreuerin / Betreuer:	Univ.-Prof. Dr. Verena Dirsch

Wien, im Juni 2009

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

Kardiovaskuläre Erkrankungen sind die häufigste Todesursache in der westlichen Welt. Angiotensin II (Ang II) begünstigt die Entstehung dieser Erkrankungen durch seine Funktion als Vasokonstriktor, Wachstumsfaktor und durch Förderung der endothelialen Dysfunktion. In glatten Gefäßmuskelzellen führt Ang II zu Zellwachstum (Hypertrophie). Es wurde bereits gezeigt, dass das im Rotwein vorhandene Polyphenol Resveratrol (RV) die Ang II- induzierte Hypertrophie durch Inaktivierung des PI3K/Akt Signalweges hemmt. Ein möglicher Mechanismus dafür wäre die Aktivierung der redoxsensitiven Phosphatase Shp2. Die Hypothese, die dieser Arbeit zugrunde liegt, besagt, dass RV Shp2 vor Inaktivierung schützt, indem es freie Sauerstoffradikalen reduziert, die nach der Ang II-Stimulation und EGF-R Transaktivierung entstehen. Eine Reduzierung dieser Sauerstoffradikale würde die Shp2 Aktivität erhalten, da Shp2 nicht durch Oxidation inaktiviert würde. Die detaillierten Ziele dieser Arbeit waren: a) zu untersuchen, ob RV die Phosphorylierung von Akt durch antioxidative Wirkung hemmt, b) die Quelle der produzierten Sauerstoffradikale zu bestimmen, die zur Akt Aktivierung unterhalb des EGF-R beitragen und c) zu klären, ob Shp2 das Zielmolekül von RV ist. In Zuge der Arbeit konnten wir einen antioxidativen Effekt von RV in unseren Zellen nachweisen, da es in der Lage war, Ang II- induzierte intrazelluläre Sauerstoffradikale und das basale Sauerstoffradikallevel zu reduzieren. Bei der Überprüfung der Redox-Abhängigkeit von Ang II- u nd EGF- induzierter Phosphorylierung und Aktivierung von Akt, p38 und ERK1/2 fanden wir, dass die Ang II vermittelte Stimulierung von Akt und p38 Nox1 abhängig ist. Die Phosphorylierung der Kinasen nach EGF Stimulation war redox-unabhängig. Mit Hilfe von nicht redoxaktiven RV-Derivaten konnten wir ebenfalls zeigen, dass RV nicht als Radikalfänger agiert, wenn es die Phosphorylierung von Akt hemmt. Wir konnten weiters keine Oxidierung von Shp2 in unseren Zellen feststellen, allerdings fanden wir Unterschiede in der Shp2 Phosphorylierung und der Rekrutierung von PI3K zu Shp2 nach Ang II- oder EGF- Aktivierung der Zellen. Dies deutet auf eine unterschiedliche Regulation von Shp2 durch Ang II oder EGF hin. Diese Studie zeigt, dass zur Hemmung der Ang II verursachten Akt Phosphorylierung und Hypertrophie in glatten Gefäßmuskelzellen die antioxidativen Eigenschaften von RV nicht notwendig sind.

A CONTENTS

A Table of Contents

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.[1, 2] 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 1*A*). 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. 1*B*). The

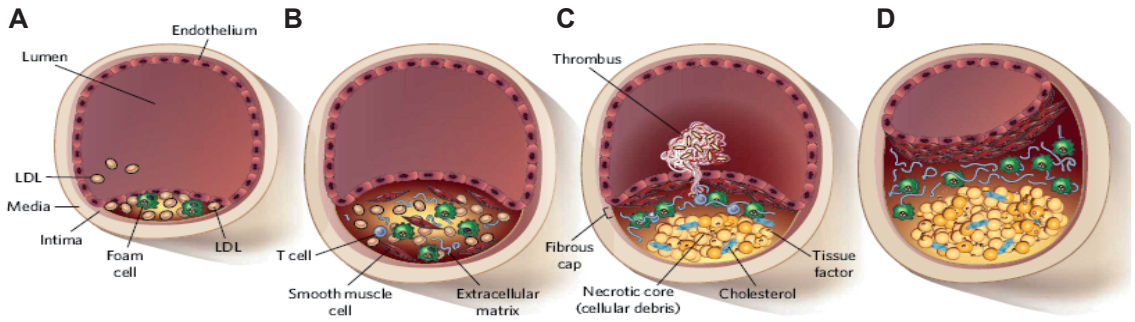

Figure 1: Initiation and progression of atherosclerosis
The first step in the development of atherosclerosis is the formation of a fatty streak (*A*), which can further lead to an intermediate lesion (*B*); then development of a lesion that is vulnerable to rupture can follow (*C*) and in the end an 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. 1*C*). 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.1*D*)

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 neointima[8].

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[9]. 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 medicine[10]. RV became famous after it was discovered in red wine, postulating that it can explain some of the cardioprotective effects of red wine[11]. The phenomenon, known as the “French Paradox”, describes the inverse correlation between red wine consumption and incidence of

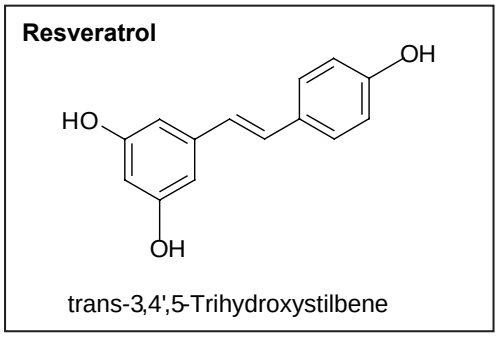

Figure 2: Structure of RV
RV possesses a *trans*-isomeric form, a stilbenic skeleton and three phenol groups.

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.[12].

2.2. Health promoting effects of resveratrol

2.2.1. Vasoprotection

RV can prevent platelet aggregation *in vitro*[13] and was also reported to block increasing platelet aggregation *in vivo*[14], further it can reduce atherosclerotic areas and the size of generated thrombi[15] by modulating COX1 over COX2 activity[16]. A vasorelaxant activity of RV was found in isolated arteries and rat aortic rings[17, 18], mediated by multiple pathways such as stimulating Ca^{2+}- activated K^+ channels and enhancing endothelial nitric oxide synthase (eNOS) as well as inducible nitric oxide synthase (iNOS)[19]. Also low-density lipoprotein (LDL) particles can be protected from oxidation by RV as it can chelate copper or scavenges free radicals[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[22]. Intracellular and extracellular ROS can be significantly inhibited at a concentration of 1-100 µM RV. Reason for this effect is that RV can scavenge free radicals[23,24] and in VSMC it was also shown to enhance endogenous antioxidants such as catalase[15,25] and to elevate glutathione, a cellular antioxidant, by influencing its viability and oxidation[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[26]. 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.[27] 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.[28] In contrast deletion of the hydroxyl group at the 4´position of RV reduced its antioxidant activity.[27, 29]

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.[9, 30] 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.[9, 31, 32] 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.[9, 31]

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[33]. 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.[33, 34] Lately RV was found to extend the lifespan of *S.cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*.[35, 36], and also of a species of short-lived fish [37]. 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[38]. Furthermore, RV was found in an initial *in vitro* screen as the most

potent of 18 inducers of SIRT1 deacetylase activity[35]. These effects of RV on SIRT1 were not reproducible when not using a specifically modified substrate *in vitro*[39], though RV was able to upregulate the Sirt1 enzyme *in vivo*.[40]

2.2.5. Resveratrol and Angiotensin II

Ang II plays an important role in controlling cardiovascular homeostasis and development of cardiovascular diseases[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.[42-44] Publications using cardiomyocytes showed that Ang II-mediated hypertrophy was inhibited by ROS attenuation of RV[45]. Furthermore, RV was able to interfere with *in vivo* cardiac hypertrophy by modulation of NO and ET-1 production.[46] In cardiac fibroblasts an Akt-independent inhibition of proliferation and differentiation after Ang II treatment was detected by RV[47]. A recent publication showed that RV attenuated Ang II-induced interleukin (IL)-6 expression via suppression of the IL-6 gene promoter activity.[49]

2.2.6. Bioavailability

Due to a rapid metabolism, RV has a short initial half-life of about 8 to 14 min[50, 51]. *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[52]. When given intravenously, RV is converted to sulphate conjugates within ~ 30 min in humans[53]. Although the concentrations of *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 ~ 27 µg/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.[9]

We used a rather high dose of RV (50 µM) 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.[54] 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.[55-57] 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).[56, 58-60] In rats chronic Ang II infusion leads to a hypertension and hypertrophy.[61]

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.[60, 62] 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 .[63-64, 65] 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.[60, 66] 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.[67, 68]

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^{2+}-dependent proline-rich tyrosine kinase 2 (Pyk2), focal adhesion kinase (FAK) and Janus kinases (JAK)).[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[69] leading to activation of several serine/threonine kinases such as PKC, MAPK kinases (ERK1/2, p38 and c-jun N-terminal kinase (JNK))[60], whereas other studies did not show a clear dependence of MAPK on ROS production[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 ($G_{q/11}$, G_i, G_{12}, G_{13}) producing second messengers including ROS.[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-triphiosphate (IP3) and diacylglycerol (DAG) are produced within seconds, leading to calcium efflux into the cytoplasm via IP3 binding and subsequently opening of Ca^{2+}-channels on the sarcoplasmic reticulum. By recruiting Ca^{2+} to myosin light chain kinase (MLCK) the interaction between actin and myosin is increased, the VSMC contract.[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.[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).[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[74]. Activation of the MAPK cascade influences cell survival, apoptosis and differentiation.[75]

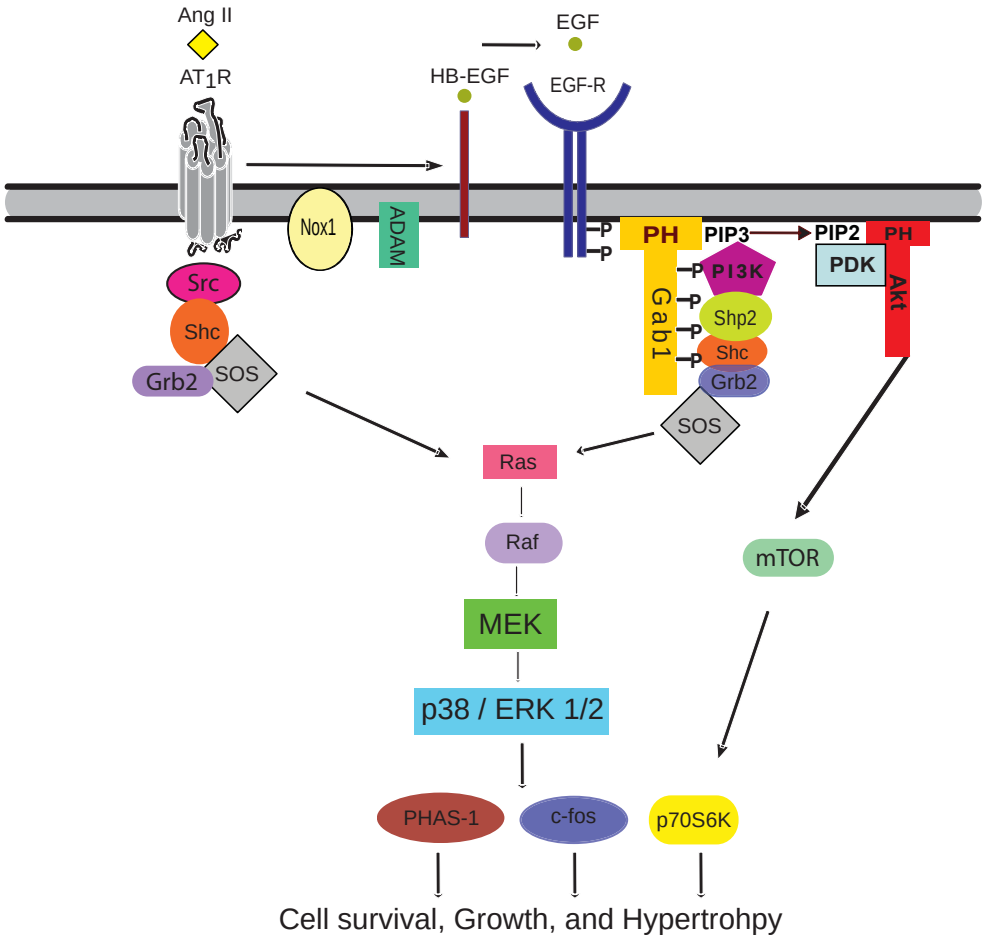

Figure 3: Simplified overview of the Ang II signaling in VSMC
Ang II regulates cell survival, growth and hypertrophy by activation of Noxes and subsequently transactivation of the EGF-R or activation of the MAPK pathway downstream of the AT1R directly. The EGF-R itself can activate the MAPK as well as the PI3K/Akt signaling pathway. Adopted from Metha P.K. et al[60] and Higuchi S. et al[72].

4. EGF and EGF-R

The family of epidermal growth factors (EGF) consists of several peptide growth factors[76], 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.[77] 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.[76] Effects of EGF

appeared to be more effective in intimal VSMC than in medial SMC[78]. 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.[76, 79]

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.[80] 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[76, 80] Also transphosphorylation of the EGF-R by Src was shown[81]. 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[76, 82, 83] 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[75]. Another pathway, whose activation through the EGF-R is well established is the PI3K dependent phosphorylation of Akt.[80]

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.[84] 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[85, 86]. 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.[87]

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[88]. In addition it was shown that also H_2O_2[89], endothelin[90], thrombin[91] , oxidized LDL[92] and mechanical stretch[93] can lead to an activation of the EGF-R in cultured VSMC[94]. The EGF-R transactivation by Ang II requires ROS[95] and upstream kinases such as c-Src or cAbl[72], occurring in cholesterol-rich domains of caveolae[96]. Also evidence for a dependence on calcium were found[60]. The activation of c-Src and cAbl lead to release of HB-EGF by metalloproteinases (ADAMs)[72]. Recently ADAM17 was identified in VMSC to be responsible for the transactivation[97]. The EGF-R is phosphorylated mainly at Tyr^{1173} and Tyr^{1068} by Ang II-induced ROS production.[70, 95] Phosphorylated Tyr^{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.[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^{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.[99] Following the receptor activation members of IR substrate (IRS) family, consisting of IRS-1 to IRS-4, are phosphorylated[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.[101] The PI3K pathway is responsible for most metabolic actions of insulin, whereas the MAPK pathway regulates gene expression and cell growth.[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[102]. In VSMC several reports show that Ang II can impair the insulin-mediated IRS-1 tyrosine phosphorylation and IRS-1/PI3K association[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[104].[99]

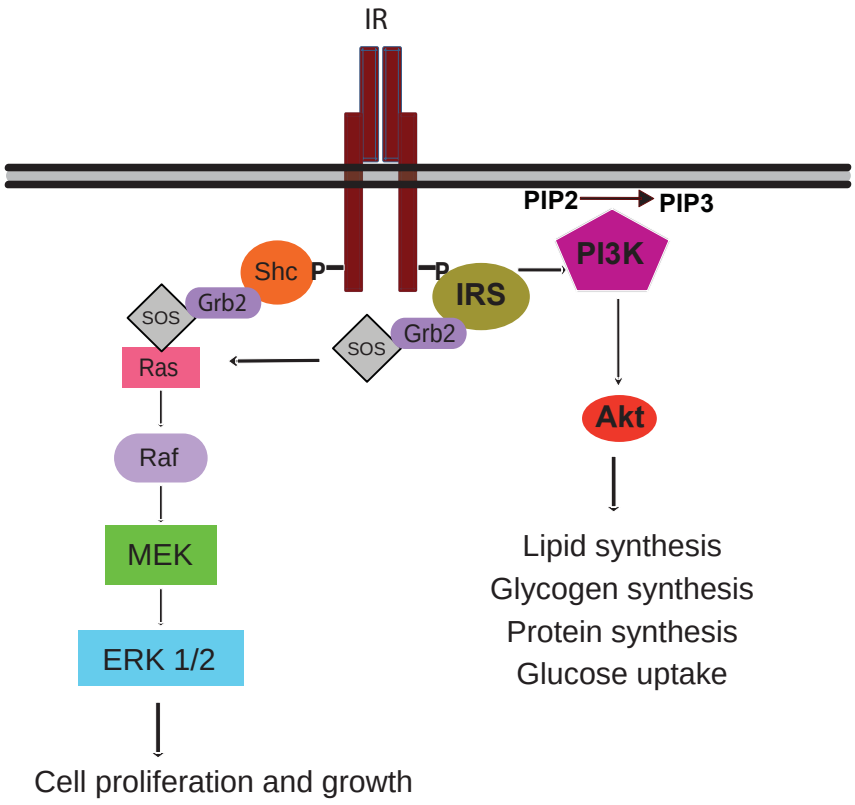

Figure 4: Insulin signaling pathway
A schematic overview of the signaling induced by Insulin binding to the insulin receptor (IR), which is leading to autophosphorylation of the receptor and subsequently recruitment of IRS and other molecules such as Shc. Activated downstream pathways contribute to lipid synthesis, glycogen synthesis, protein synthesis and glucose uptake. Adopted from Olivares-Reyes J.A. et al[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[83]. As a kinase, it is able to phosphorylate the 3´-OH position of the inositol ring of phosphoinositides[105]. 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[106]. The PI3K consists of a regulatory adapter subunit (p85) coupled to a catalytic subunit (p110α, β, γ)[107] 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[105]. In the EGF-R signaling PI3K is recruited to tyrosine phosphorylated adaptor protein Gab1, which is bound to Tyr^{1068} and Tyr^{1086} 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. [108,83]

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[109]. 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[110]. 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[106, 109]. 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 Tyr^{308}. In addition, also the so called kinase PDK2 is recruited which phosphorylates Akt

at Ser[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.[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 a inactive status. Upon phosphorylation by Akt GSK3 is inactivated leading to cell cycle progression and protein synthesis[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[115] as well as BAD activation[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[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).[109] Protein synthesis is regulated by Akt via mTOR dependent p70S6 kinase activation, inducing transcription factors AP-1 and E2F.[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.[118-120] Extracellular-signal regulated kinases (ERKs), p38MAPK (p38α, p38β, p38γ, p38δ) 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.[121] Ras activates Raf1 which then phosphorylates ERK1/2 on a threonine and tyrosine residue.[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[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[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 α/β MAPK and JNK 1/2, promoting neointima

formation and VMSC proliferation.[124, 125] 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.[122, 126]

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 fare as the phosphatase that acts as a proto-oncogene. [127] Phosphatases are also related to many other diseases including autoimmunity and developmental disorders.[128] 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 HCX_5R, including a cystein residue which is a nucleophile and essential for catalysis. [128-131] Due to their microenvironment the catalytic cysteins ($C\text{-}S^-$) 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 cystein 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 ($C\text{-}SO_2H$) or sulphonic ($C\text{-}SO_3H$) acids are usually irreversible.[132-134] 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[130] or Shp2[135]. Transactivation of a receptor, like the Ang II induced activation of the EGF-R is often combined with H_2O_2 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.[130, 136, 137] This tight regulation is achieved by a localized ROS production specifically inactivating a single phosphatase and varying among cells.[131]

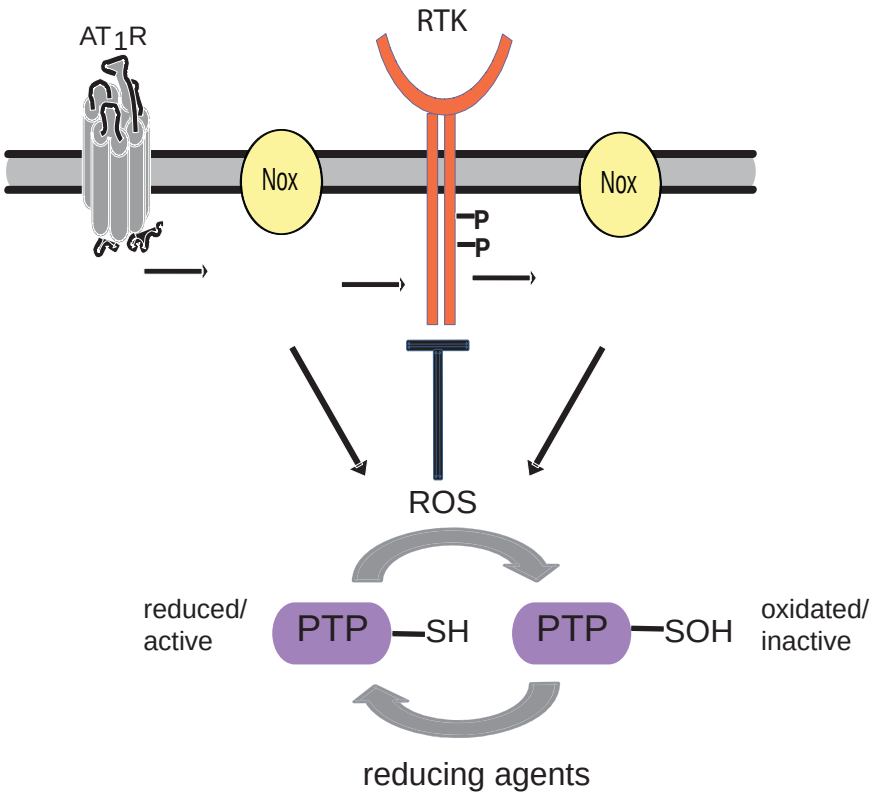

Figure 5: ROS-mediated modulation of receptor tyrosine kinases (RTK) and protein tyrosine kinases (PTPs) in Ang II signaling
Tyrosine phosphorylation and subsequently activation of RTKs like the EGF-R can be promoted by ROS via inactivation of PTPs, leading to its activation. PTPs are catalytically inactivated by ROS-induced oxidation which can be reversed by reducing agents. Adopted from Chiarugi P. et al[138].

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 paralogue 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.[139, 140] 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.[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.[142]

9.1.1. Posttranslational modification of Shp2 - oxidation and phosphorylation

The main ways of regulation of Shp2 are phosphorylation at the residues Tyr^{542}, Tyr^{580}, Ser^{576} and Ser^{591}, oxidation of the cystein residue at the catalytic site[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[139]. By binding of the SH2 domains to a tyrosine phosphorylated ligand the PTP activity increases dramatically.[130] The two tyrosine residues (Tyr^{542}, Tyr^{580}) are then susceptible to

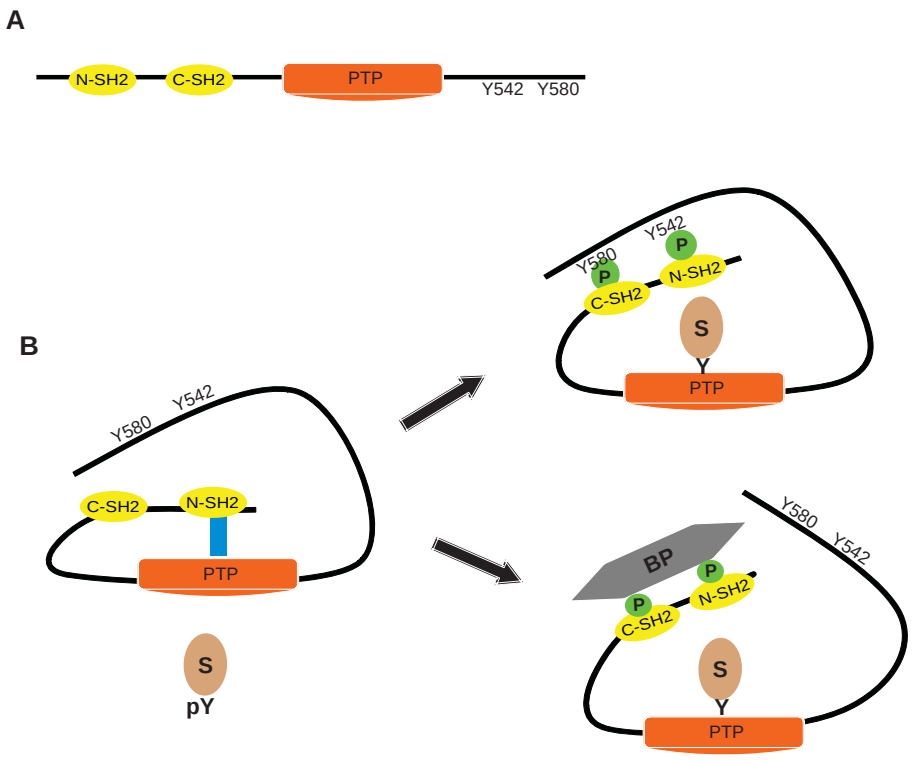

Figure 6: Shp2 structure and regulation
A, *Schematic structure of Shp2.* Shp2 contains two SH2 domains, a N-terminal and a C-terminal SH2 domain, a catalytic PTP domain and a C-terminal tail containing two tyrosine residues. *B*, *Potential mechanism of Shp2 regulation.* In basal state Shp2 is largely 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^{542} and Tyr^{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.[143] and Shen K.[139]

phosphorylation by different receptors and non-receptor protein tyrosine kinases (PTK) and other proteins including insulin response substrate-1 (IRS-1), and Gab1.[143] 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[140]. PKC activation leads to a serine phosphorylation of Shp1 and Shp2, but Shp2 activity seems not to be affected by this action.[144] 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.[145] Currently, it is unclear which of the tyrosine residues of Shp2 are important for the activity, as contradictory data is found in the literature.[146, 147] 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.[148] A recent publication indicates a redox-dependent regulation of Shp2 by Ang II via oxidation and phosphorylation in VSMC.[149]

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.[150, 151] 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 Tyr^{922}, 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.[121, 152] Later it was shown that this effect is mediated by dephosphorylation of the binding site for RasGAP on Gab1 by Shp2.[153] 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.[154] Activation of Shp2 by RV was shown in fibroblasts by our own group previously.[44] 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.[155] Recently also an interaction of c-Src and Shp2 was detected in endothelial cells after Ang II stimulation, influencing Nox and ERK1/2 activity.[156]

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.[157,158] Members of the ROS family normally contain unpaired electrons. The superoxide anion ($O_2^{\bullet -}$) is the so called "primary" ROS; produced in the vessels mainly by NADPH oxidases, the "secondary" ROS include hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and nitric oxide (NO).[159, 160] Other sources of ROS in VSMC are xanthine oxidase, lipoxygenase and nitric oxide synthase. H_2O_2 is cell-permeant, very stable and generated primarily by superoxide dismutase (SOD)-mediated dismutation of $O_2^{\bullet -}$.[161, 162] Intracellular regulators of ROS are catalase and glutathione peroxidase (Gpx1). Catalase is found mainly in peroxisomes, catalysing the reaction of H_2O_2 to water, Gpx1 is located in the cellular cytosol and mitochondria, favouring also the detoxification of H_2O_2 to water by producing glutathione disulfide out of glutathione.[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[163]. H_2O_2 derived from NADPH oxidases was shown to be needed for the activation of c-Src[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.[161] H_2O_2 treatment was shown to induce the phosphorylation of Akt[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^{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.[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.[170,171] Table I shows the expression of different Noxes in tissues.[168, 172, 173]

	Activation	***High level of expression***
Nox1	inducible	colon epithelial cells, VSMC, endothelium
Nox2	inducible	phagocytes, endothelium, cardiomyocytes
Nox3	constitutively active ?	inner ear, fetal kidney, fetal spleen
Nox4	constitutively active	endothelium, VSMC, kidney, osteoclasts
Nox5	inducible	lymphoid tissue, endothelium, VSMC,

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.[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.[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^{2+} dependent and contain a peroxidase-homology domain. This enables Duox to both generate reactive oxygen and use it

through its own peroxidase domain[169, 171, 175] In animals Nox1-Nox4 form a heterodimer with the membrane protein p22phox, a complex known in phagocytes as flavocytochrome b_{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.[165, 169]

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.[162,176] The main ROS species produced by this pathway is superoxide ($O_2^{\bullet-}$)[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.[172] In VSMC the involvement of p67phox or NOXA in Nox1 activation is not well defined in literature.[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.[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.[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.[181] A role for PI3K in Rac-1 activation is suggested since Ang II-triggered ROS release can be inhibited by PI3K inhibitors.[164] Finally, this localized ROS production seems to be necessary for EGF-R transactivation.[178]

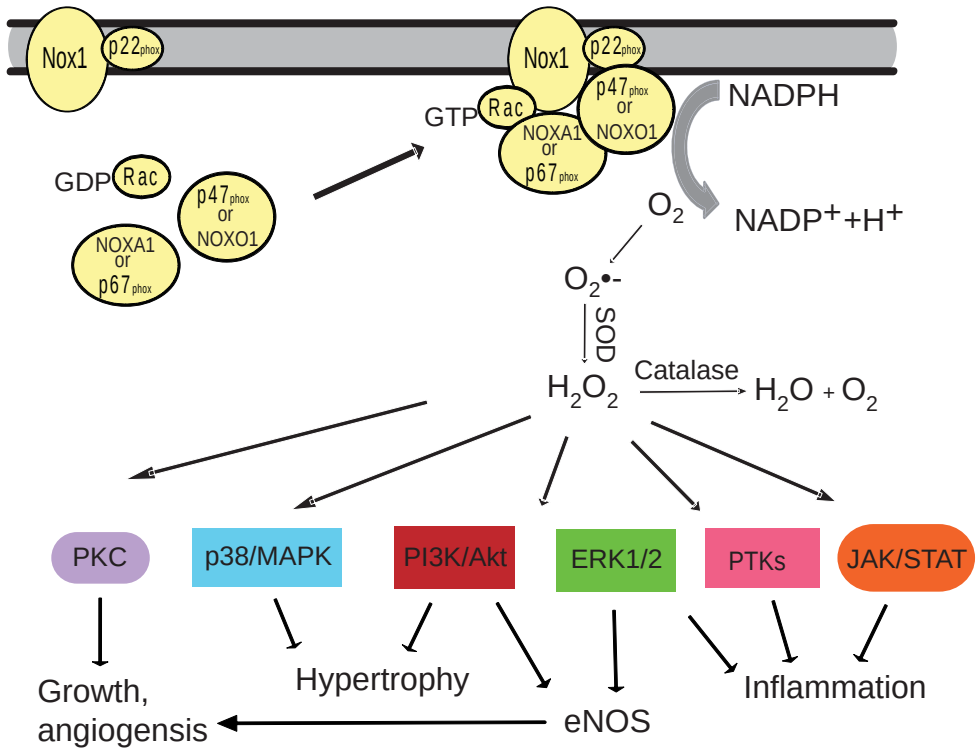

Figure 7: Structure and activation of Nox1 in VSMC
For the activation of Nox1 in VSMC the cytosolic subunits p47phox (NOXO), Rac and possibly also NOXA (p67phox) have to be translocated to the two membrane-bound subunits Nox1 and p22phox. The activation results in the formation of superoxide ($O_2^{\bullet-}$), which is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). The intracellular antioxidant catalse can decompose H_2O_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 activation[186].[165, 171] Nox1 knock out mice were shown to develop less Ang II-induced hypertension[187], whereas conflicting observations were made concerning Nox1 involvement in hypertrophy.[187, 188]

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 [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. [190] In contrast to other Noxes, the main ROS produced by Nox4 after Ang II treatment is hydrogen peroxide (H_2O_2).[177]

Studies show that after vascular injury, during hypoxia and serum deprivation the Nox4 protein expression was strongly induced.[171, 184] Other data indicated that stimuli like Ang II, thrombin and PDGF induce Nox1 expression but down regulate Nox4 expression level.[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.[95, 181, 191] When the EGF-R and cave olin-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.[178]

12. Aim of the work

In the western world cardiovascular disease (CVD) is the leading cause of death.[2, 192] 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.[55] In vascular smooth muscle cells (VSMC), Ang II promotes cell growth (hypertrophy).[54] It was recently shown that resveratrol (RV) inhibits Ang II-induced hypertrophy in VSMC by interfering specifically with the PI3K/Akt pathway [42]. 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)[44]. 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[154] (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.

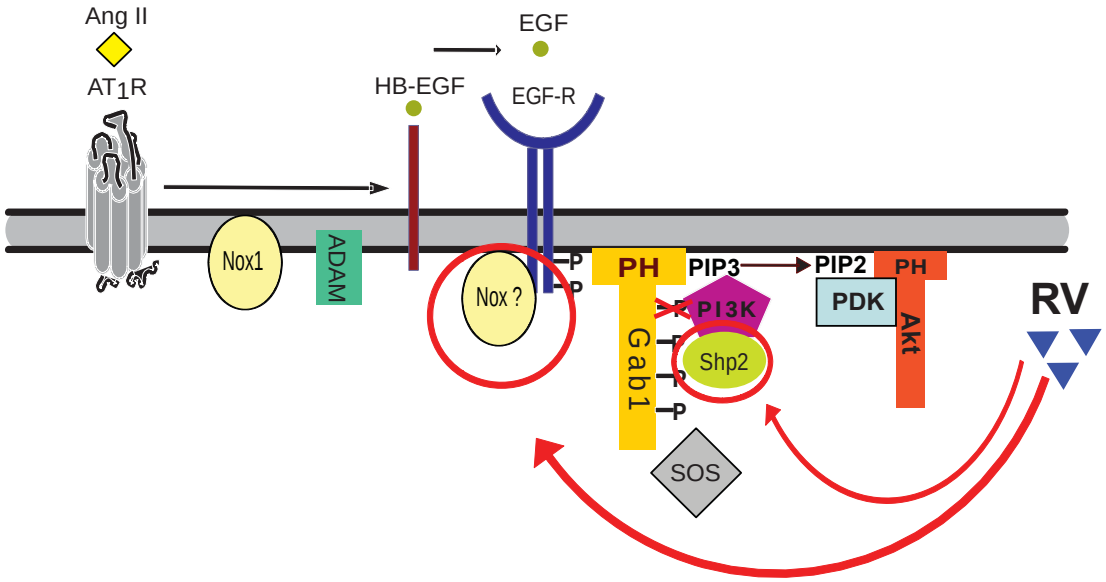

Figure 8: Hypothesis

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.[154] 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 cm^2 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 % CO_2 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 cm^2 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 10^6 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 75cm^2 flasks.

Cell culture media and reagents

Name	***Provider***
Dulbecco's Modified Eagle Medium (DMEM), 5 g/L glucose, w/o L-glutamine, w/o phenolred	Lonza Group Ltd. (Basel, Switzerland)
Penicillin/streptomycin mixture (10,000 U/ml potassium penicillin/ 10,000 µg/ml streptomycin sulphate)	Lonza Group Ltd. (Basel, Switzerland)
L-glutamine 200 mM	Lonza Group Ltd. (Basel, Switzerland)
Trypsin/EDTA (1:250)	Invitrogen (Carlsbad, CA, USA)
Calf serum	Lonza Group Ltd. (Basel, Switzerland)

Table II: Cell culture reagents

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

Solutions for cell culture

Solutions: cell culure	*Reagents*	
PBS pH 7.4	NaCl	7.2 g
	Na_2HPO_4	1.48 g
	KH_2HPO_4	0.43 g
	ddH_2O	ad 1000 ml
PBS$^+$	NaCl	8.0 g
	KCl	0.2 g
	Na_2HPO_4	1.15 g
	KH_2HPO_4	0.2 g
	$MgCl_2$ x 6 H_2O	0.1 g
	$CaCl_2$ x 2 H_2O	0.1 g
	ddH_2O	ad 1000 ml
Growth medium	DMEM	500 ml
	Calf Serum (CS)	10 %
	Penicillin/Streptomycin	100 U/ml/100 µg/ml
	L-glutamine	2 mM
Starving medium	DMEM	500 ml
	Calf Serum (CS)	0.1 %
	Penicillin/Streptomycin	100 U/ml/100 µg/ml
	L-glutamine	2 mM
Trpysin/EDTA in PBS (T/E)	Trypsin	0.05 %
	EDTA	0.02 %
Freeze medium	DMEM	8.0 ml
	Calf Serum (CS)	2.0 ml
	DMSO	1.1 ml
Digestion buffer	Collagenase (246 U/mg)	0.1 g
	HEPES	0.24
	Ascorbic acid	0.005 g
	Gentamicin sulphate	0.005 g
	BSA	0.1 g
	Ham's F12 medium (PAN^{TM} biotech)	

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 10^5 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 H_2O_2 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

Name	*Concentration*	*Provider*
AG 1478 (Tyrphostin)	250 nM	Sigma
AEBSF	10 µM - 1 mM	Sigma
DMTU	50 µM - 10 mM	Sigma/Fluka
DPI	10 µM	Sigma
ds-tat control	100 µM	Sigma/Caslo
EUK 134	50-200 µM	Cayman
gp91 ds-tat	100 µM	Caslo
Indirubin-3'-monoxime	3 µM	Laurent Meijer (Roscoff)
MnIII TM	50-200 µM	Cayman
MPG	50-300 µM	Sigma
NAC	10 mM	Sigma
Sirtinol	10 µM	Sigma
Wortmannin	50 nM	NEB

Table IV: Inhibitors and antioxidants

Blocking peptides

Nox1	*Sequence*
gp91 ds-tat	[H]-RKKRRQRRRCSTRIRRQL-NH2
ds-tat control	[H]-RKKRRQRRRAGAGAGAGA-NH2

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

Solutions	*Reagents*	
Lysis buffer (stock solution)	HEPES	50 mM
	NaCl	50 mM
	NaF	50 mM
	$Na_4HP_2O_7$ x 10 H_2O	10 mM
	EDTA	5 mM
	Na_3VO_4	1 mM
	ddH_2O	add 430 ml, pH 7.5
Prior to use	Stock solution	4.3 ml
	PMSF 0.1 M	50 µl
	Complete™ 25x	202 µl
	Trition X-100 (10%)	500 µl
WB sample buffer stock	Tris-HCl (0.5 M; pH 6.8)	37.5 ml
	SDS	6.0 g
	Glycerol	30 ml
	Bromphenol blue	15.0 mg
3x sample buffer	WB sample buffer stock	850 µl
	2-mercaptoethanol	150 µl

Table VI: Buffers for protein lysates

3.1. Protein quantification

The amount of protein was determined using the method of Bradford.[193] The protein lysates were diluted in ddH_2O 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.[194] 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.

Gels, buffers and reagents for SDS-PAGE and Western blotting

Solutions	*Reagents*	
Resolving gel (10 % PAA)	Roti®-Phorese Gel 30	5 ml
	ddH2O	6.10 ml
	Tris-HCl (1.5 M; pH 8.0)	3.75 ml
	SDS 10 %	0.15 ml
	TEMED	15 µl
	Ammonium persulfate 10 %	75 µl
Stacking gel	Roti®-Phorese Gel 30	1.7 ml
	ddH2O	7.0 ml
	Tris-HCl (1.25 M; pH 6.8)	1.0 ml
	SDS 10 %	0.1 ml
	TEMED	20 µl
	Ammonium persulfate 10 %	100 µl
5x Electrophoresis buffer	Tris-base	15 g
	Glycine	72 g
	SDS	5 g
	ddH2O	ad 1000 ml
5x Blotting buffer	Tris-base	15.2 g
	Glycine	72.9 g
	ddH2O	ad 1000 ml
Blotting buffer	5x blotting buffer	200 ml
	Methanol	200 ml
	ddH2O	ad 1000 ml
TBS-T pH 8.0	Tris-base	3.0 g
	NaCl	11.1 g
	Tween-20	1 ml
	ddH2O	ad 1000 ml
Home-made ECL freshly prepared prior to use	ddH2O	4.5 ml
	Tris-base (1 M; pH 8.5)	0.5 ml
	Luminol (0.25 M in DMSO)	25 µl
	p-coumaric acid (90mM in DMSO)	11 µl
	H2O2 30 %	3 µl

Table VII: Gels, buffers and reagents for protein seperation and detection

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

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

Table VIII: antibodies and beads

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

5. ROS detection

5.1. Intracellular ROS detection with FACS

Intracellular ROS was detected using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA).[195] 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_2DCF) 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_2DCF-DA final concentration per well. Cells were stimulated after additional 15 min and then the buffer was aspirated, cells were trypsinized

Solutions and buffers for ROS measurement

Solutions	*Reagents*	
FACS buffer pH 7.37	NaCl	8.12 g
	KH_2PO_4	0.26 g
	Na_2HPO_4	2.35 g
	KCl	0.28 g
	LiCl	0.43 g
	NaN_3	0.20 g
	Na_2EDTA	0.36 g
	ddH_2O	ad 1000 ml
PBS/BSA 2 %	BSA dissolved in PBS	
HBSS Buffer	NaCl	8.18 mg
	KCl	400 mg
	Na_2HPO_4	19.75 mg
	KH_2PO_4	50 mg
	$CaCl_2$ x H_2O	180 mg
	$MgSO_4$	97.5 mg
	Glucose	1000 mg
	HEPES	4760 mg
	ddH_2O	ad 1000 ml

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 Fl1h 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 resorufin in the presence of H_2O_2 in a 1:1 stoichiometry[196].

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 resorufin 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.[197,198] 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 sulfenic 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.

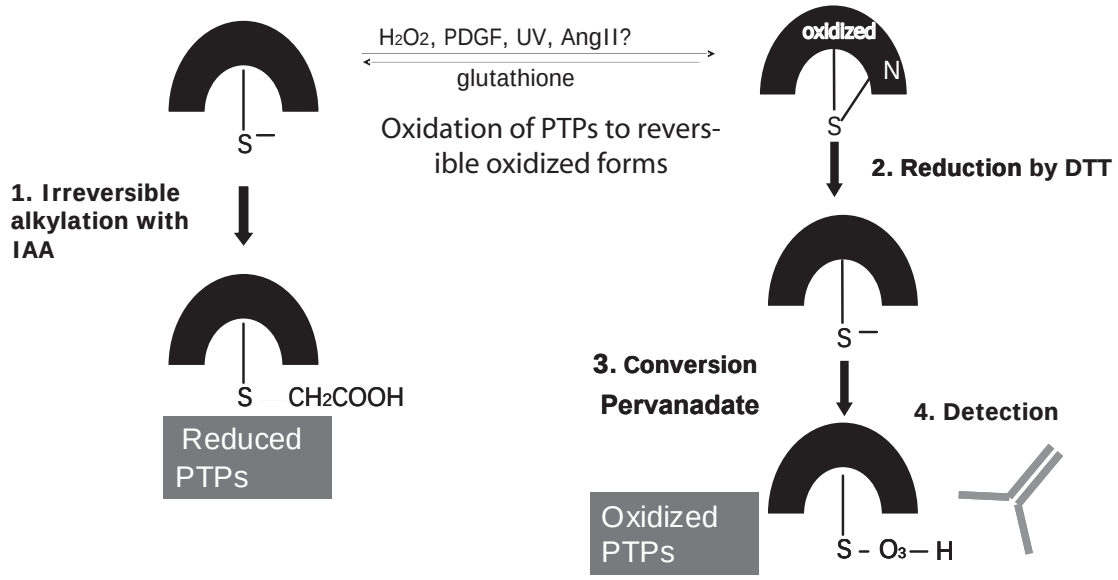

Figure 9: Antibody-based oxidized PTP detection assay
Schematic overview of the method to detect oxidized PTPs with a specific antibody. After stimulation all reduced PTPs are protected for further oxidation by IAA treatment. Only oxidized PTPs are then reduced with DTT and converted from a sulfenic 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[197].

3.5 x 10^6 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 Hepes 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 Hepes buffer. After that, lysates were treated with freshly prepared Pervanadate (100 mM) at a final concentration of 100 µM for 1 h. Samples were

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 (Invirtrogen) 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

Name		*Sequence*	
Nox4:		5-'ACUGAGGUACAGCUGGAUGUU-3' 5-'CAUCCAGCUGUACCUCAGUUU-3'	sense anti-sense
Shp2:	Seq 1	5-'UACUUGACACACUUGCUCUUCCCUC-3' 5-'GAGGGAAGAGCAAGUGUGUCAAGUA-3'	sense anti-sense
	Seq 2	5-'GGACUGUGACAUCGACGUUCCUAAA-3' 5-'UUUAGGAACGUCGAUGUCACAGUCC-3'	sense anti-sense
	Seq 3	5-'UCCACCAGGGAAUACUUAAUAUUGG-3' 5-'CCAAUAUUAAGUAUUCCCUGGUGGA-3'	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

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

Table XII: Buffers for RNA gel

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

RNA concentration (µg/mg) = $Absorbance_{260}$ x 40 x 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 µg RNA , 10 ng random hexamers/µg RNA, 1 µl 10 mM dNTP mix (deoxyribonucleotides), 1 µl luciferase RNA (10^8 copies, exogenous standard) and filled to a total volume of 12 µl with RNase-free water. Samples were incubated at 65 °C for 5 min for denaturation, left on ice for 1 - 2 min and 4 µl 5x first strand buffer, 2 µl DTT (0.1 M) and 1 µl RNaseOUT inhibitor (40 U/µl) were added. This mix was left at room temperature for 2 min in order to allow primers to anneal. Then 1 µl Superscript™ II RT enzyme (50 U/µl) 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 µl RNase H was added to each tube for digesting remaining RNA. Finally the cDNA was purified using Bio-Spin® 30 Tris Colums (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.

Primers for PCR

Gene	*Direction*	*Sequence*
Nox1	fwd	CTG CTC TCC TTC CTG AGG GGC ACC TGC T
	rev	GAC AAT CCC CCC CAG GCC ATG GAT CCC TA
Nox4	fwd	CCG GGC CTG ACA GGT GTC TGC ATG GTG
	rev	CAC AGT ATA GGC ACA AAG GTC CAG AAA TCC AAA TCC AGG T
18 S	fwd	GAA TTG ACG GAA GGG CAC CAC CAG
	rev	GTG CAG CCC CGG ACA TCT AAG G
Luciferase	fwd	GAA TTG ACG GAA GGG CAC CAC CAG
	rev	GTG CAG CCC CGG ACA TCT AAG G

Table XIII: Sequence of primers used for PCR

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

D Results

1. Characterisation of VSMC

Subcultured VSMC develop a proliferative phenotype resembling the cells in the neointima[8]. 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.[42, 44] 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.[199] 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).

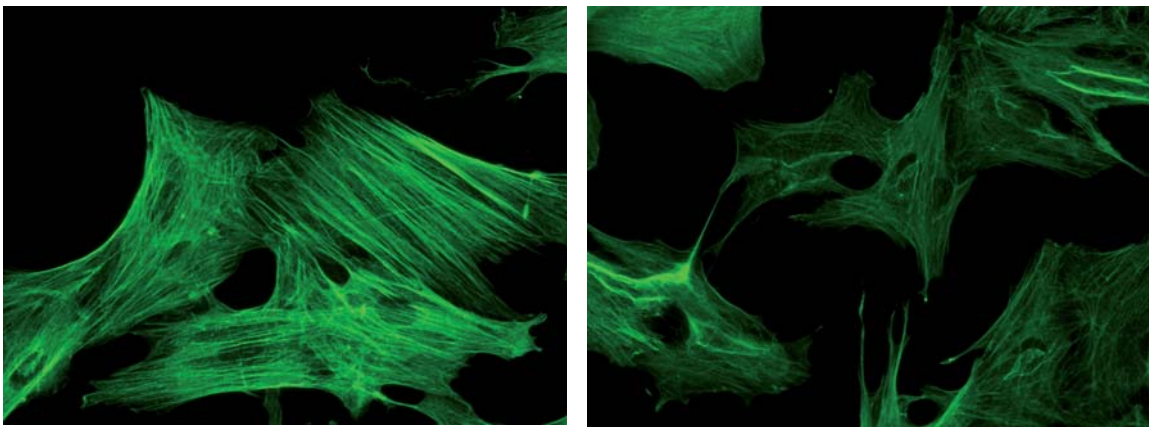

Figure 10: Anti-α-smooth muscle actin staining of VSMC
Image shows VSMC immunostained with FITC-conjugated anti-α-smooth muscle actin antibody in 400-fold magnification.

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.[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. 11*A*) or EGF (100 ng/ml) for 5 min (Fig. 11*B*) 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**
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[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.[44] A suggested mechanism for

the antioxidative effect of RV would be a modulation of Noxes, potential ROS sources in VSMC. Therefore we used diphenylene iodium (DPI) as an unspecific inhibitor of flavin-containing enzymes including NADPH oxidases, xanthine oxidases and cytochrome P450 enzymes[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[186] or by transactivation of the EGF-R through Nox dependent ROS[60, 95].

To test, whether RV can inhibit Ang II- or EGF-mediated ROS production we loaded the cells with 20 µM H_2DCF-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*A*) and also preincubation with DPI, the positive control, inhibited ROS in accordance to literature.[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*B*), again verifying a strong antioxidant effect for both compounds.

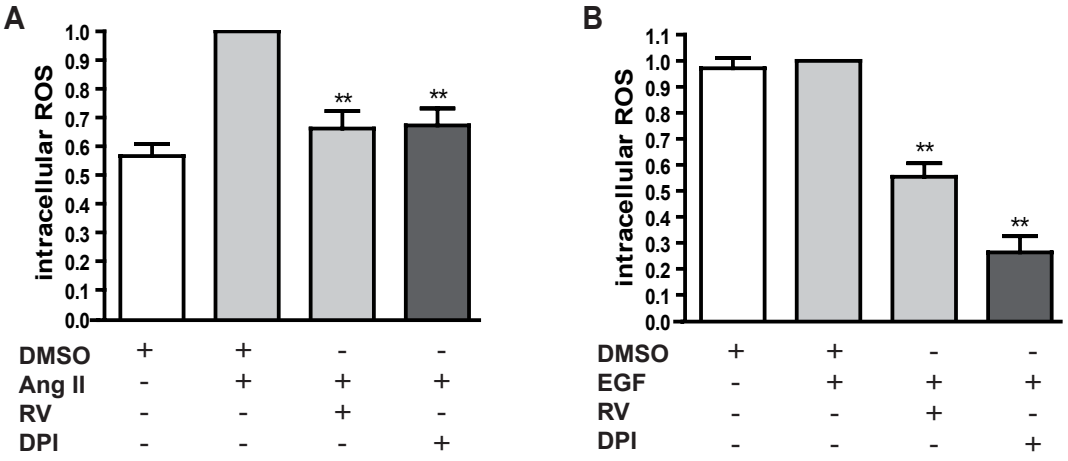

Figure 12: RV diminishes intracellular ROS level after Ang II and EGF stimulation
Quiescent VSMC were incubated 15 min with 20 µM H_2DCF-DA in HBSS buffer, then they were preincubated with 50 µM RV or 0.1 % DMSO (vehicle control) for 30 min or 10 µM DPI for 1 h and finally stimulated with 100 nM Ang II for 15 min (*A*) or with 100 ng/ml EGF for 10 min (*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).

Upon EGF stimulation it was shown that H_2O_2 was produced extracellular after EGF-R transactivation, also influencing phosphatases.[203] Examining extracellular H_2O_2 production in VSMC we applied an Amplex Red™ assay (performed by Mario Kumerz, Dissertation 2009, Uni. Vienna), which is detecting extracellular produced H_2O_2.

In accordance with our intracellular ROS data we also observed a high level of extracellular H_2O_2 produced by unstimulated VSMC. Stimulation with Ang II and EGF was able to induce this production of extracellular H_2O_2 in a time-dependent manner (Fig. 13 *A*-*B*). EGF treatment led to a peak of H_2O_2 after 15 min (20 % above basal level). H_2O_2 production was highest 10 min after Ang II stimulation (15 % above basal level) but declined at later time points.

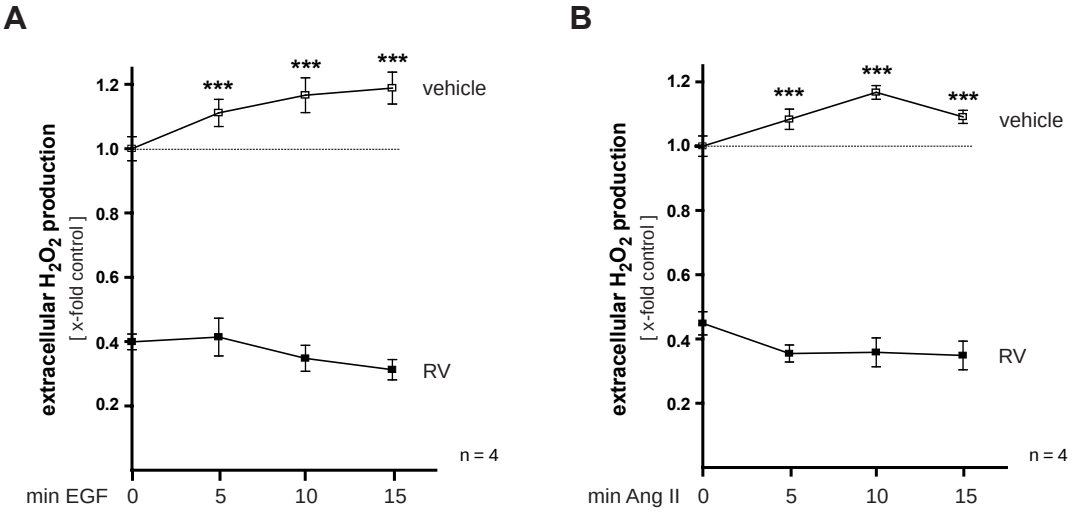

Figure 13: RV extenuates both basal and Ang II- or EGF-induced extracellular H_2O_2 levels

(*A*) Serum starved VSMC were stimulated in Amplex Red buffer with EGF (100 ng/ml) for 5 to 15 minutes after preincuabtion with RV or vehicle control for 30 minutes. H_2O_2 production was quantified using a fluorometer. Absolute values were correlated to catalase-treated cells used as negative control. Graph shows relative extracellular H_2O_2 production expressed as x-fold vehicle-treated cells (–□–, vehicle-treated cells; –■–, RV-treated cells; ***, $p < 0.001$; mean ± 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; ***, $p < 0.001$; mean ± 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[204] 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)[48]. 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[162, 168]. Nox4 was detected in focal adhesions of VSMC where also the EGF-R is localized after tyrosine phosphorylation[96, 178, 205]. In Ang II signaling in VSMC ROS production by Nox1 is suggested as the major component for the transactivation of the EGF-R[95, 164], 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

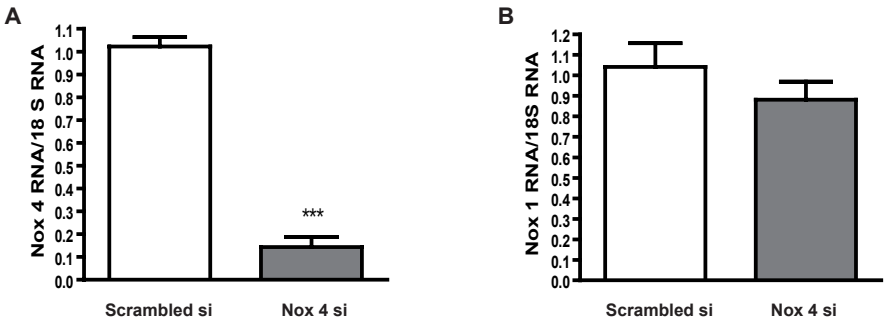

Figure 14: Specific down-regulation of Nox4 in VSMC
Knock down was achieved by treating the cells with 50 µM siRNA against Nox4 or 50 µM scrambled control for 72 h. mRNA was isolated and expression of Nox4 was detected with real-time PCR. Graphs show mean Nox4 mRNA (*A*) or Nox1 mRNA (*B*) level relating to 18S mRNA levels of three independent experiments. Data were normalized setting scrambled control to 1 (***, $p < 0.001$; t-test).

was detected (Fig. 14*A*). In contrast, the mRNA level of Nox1 was not impaired by Nox4 siRNA treatment (Fig. 14*B*), 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. 15*A*). The phosphorylation level of p38 (Fig. 15*B*) and ERK 1/2

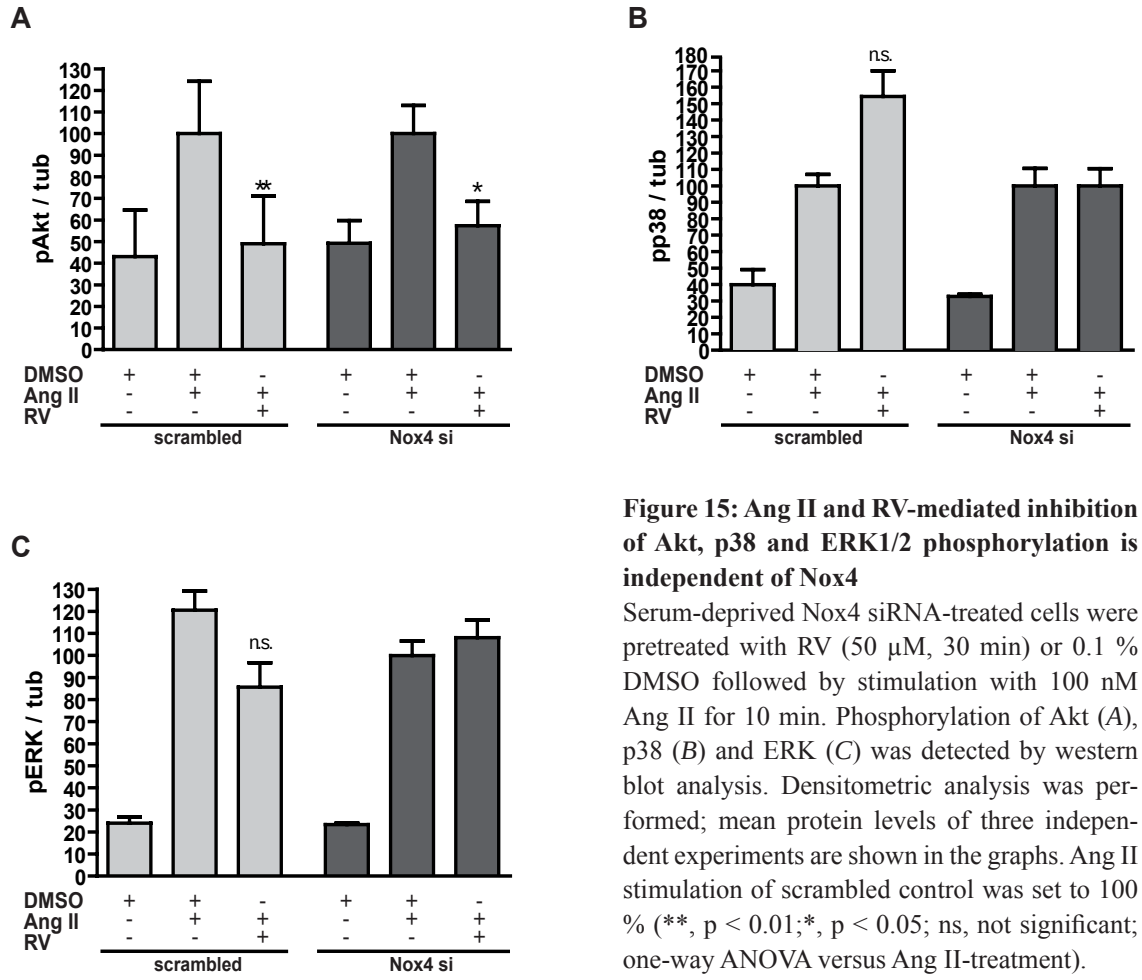

Figure 15: Ang II and RV-mediated inhibition of Akt, p38 and ERK1/2 phosphorylation is independent of Nox4

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. 15*C*) 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. 16*A*) as well as phosphorylation of p38 (Fig. 16*B*), and ERK1/2 (Fig. 16*C*) were analyzed by

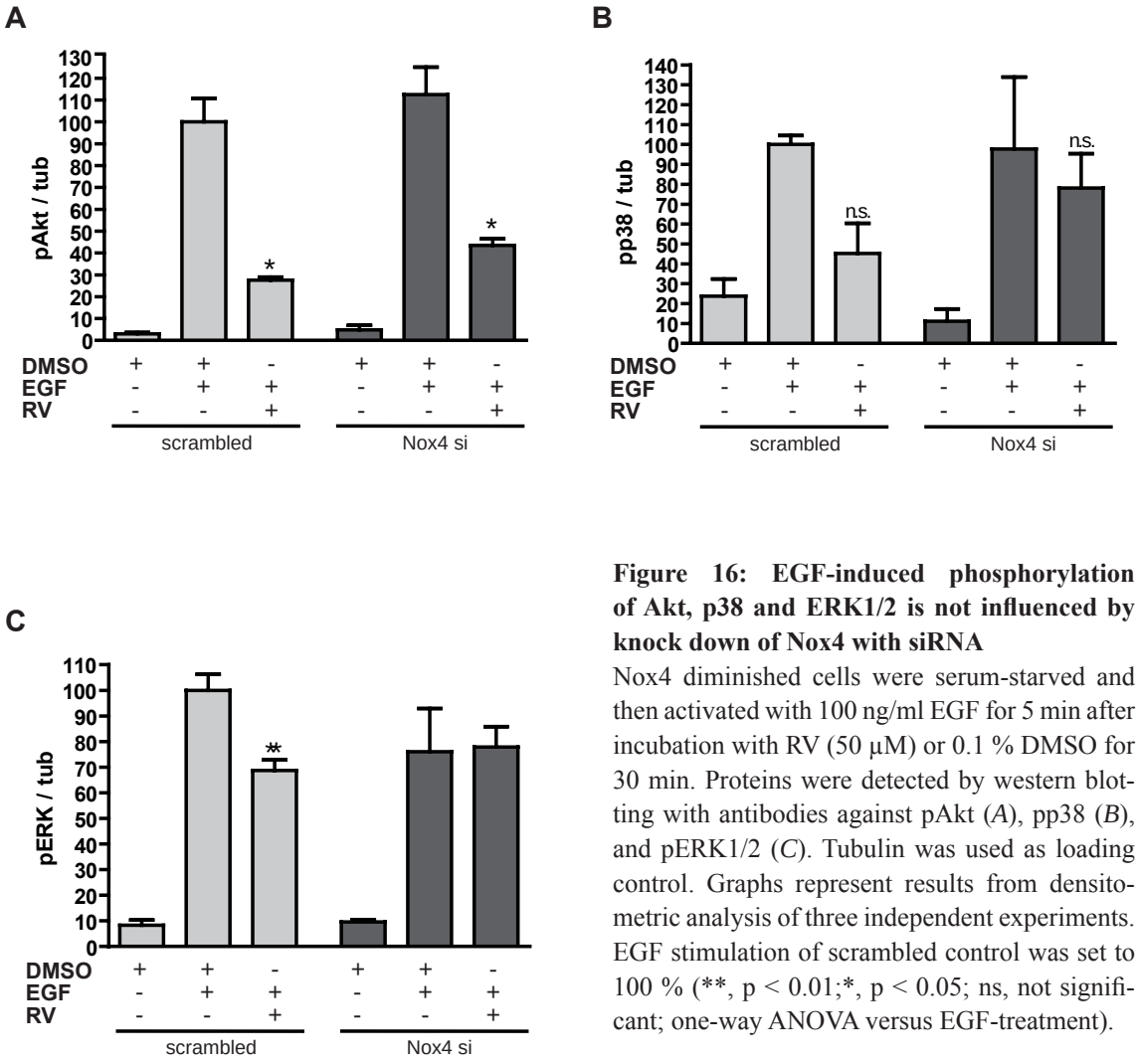

Figure 16: EGF-induced phosphorylation of Akt, p38 and ERK1/2 is not influenced by knock down of Nox4 with siRNA
Nox4 diminished cells were serum-starved and then activated with 100 ng/ml EGF for 5 min after incubation with RV (50 µM) or 0.1 % DMSO for 30 min. Proteins were detected by western blotting 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. 17*A*-*B*). In contrast to RV, gp91ds-tat peptide inhibited p38 phosphorylation significantly by more that 50 % (Fig. 17*C*) but phosphorylation of ERK only showed at slight but not significant trend of reduction (Fig. 17*D*). The control peptide did not influence the phosphorylation of Akt, p38 and ERK (Fig. 17*A*).

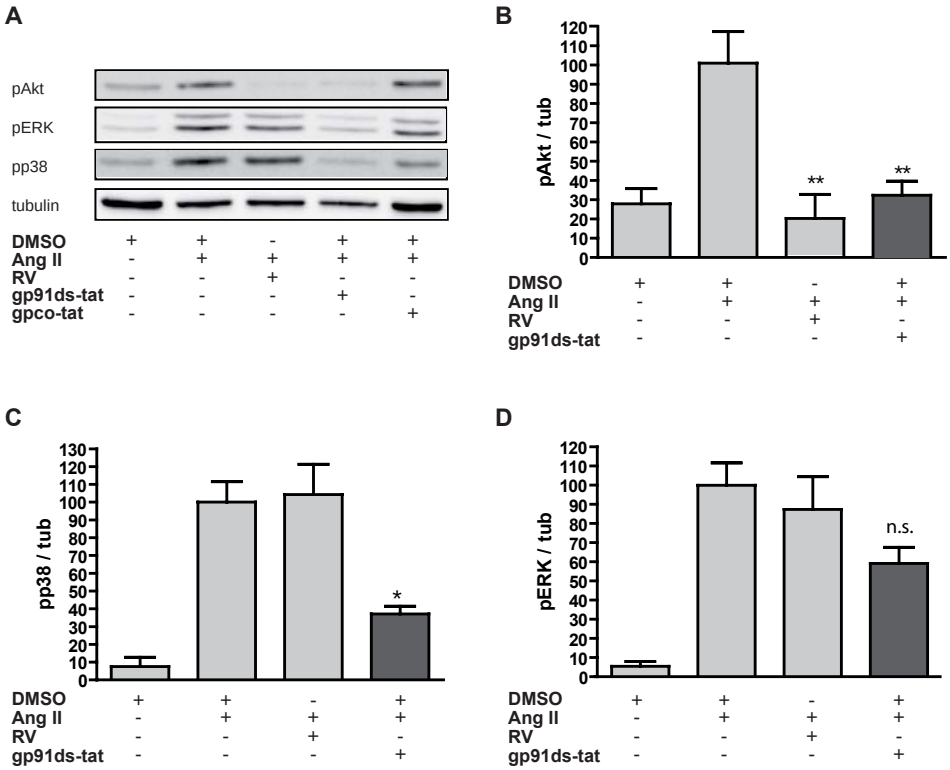

Figure 17: Nox1 inhibition blunts the phosphorylation of Akt and p38 induced by Ang II

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. 18*A-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.

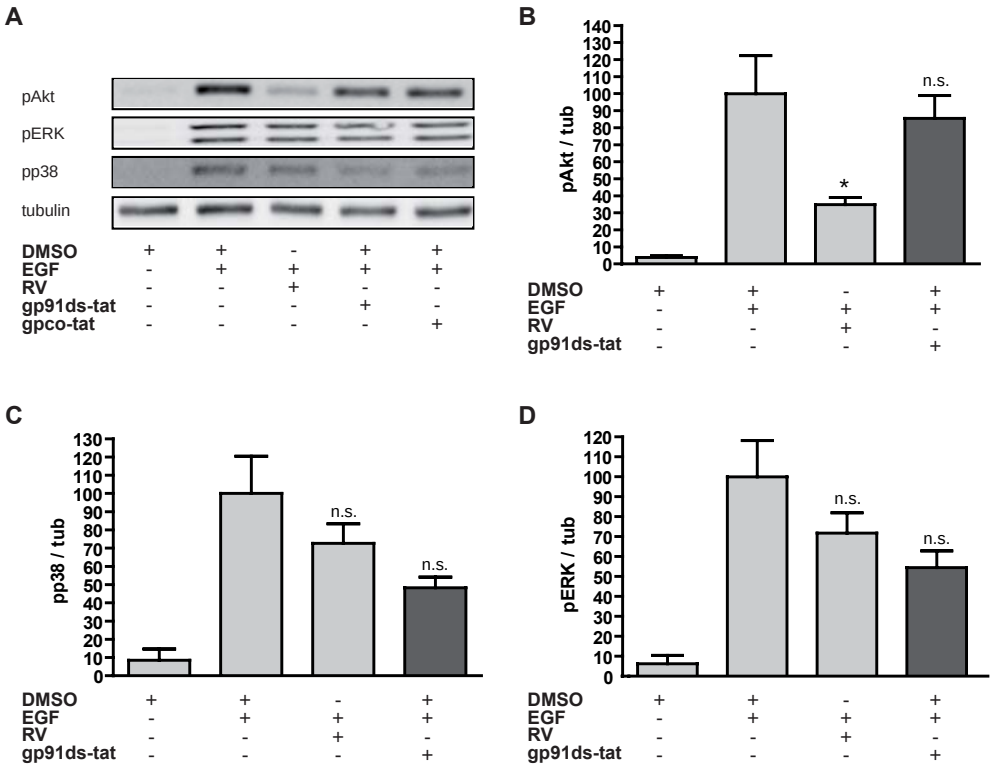

Figure 18: Phosphorylation of Akt, p38 and ERK after EGF stimulation is independent of Nox1

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[208]. AEBSF is a serine protease inhibitor that blocks the recruitment of p47phox to the Noxes enzymes[172] and can be therefore used as Nox1 inhibitor.

Cells were treated with AEBSF for 30 min, then loaded with 20 µM H_2DCF-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).

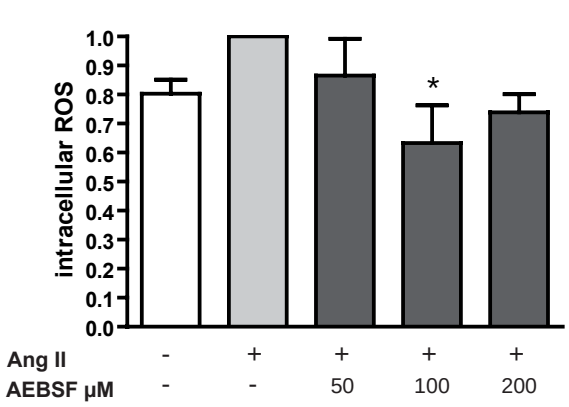

Figure 19: AEBSF treatment decreases intracellular ROS after Ang II stimulation
Serum-starved VSMC were preincubated for 30 min with AEBSF (50 µM, 100 µM, 200 µM), incubated 15 min with 20 µM H_2DCF-DA in HBSS buffer and then cells were stimulated with 100 nM Ang II for 15 min. Using flow cytometry intracellular 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).

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[209] or 2 h before stimulation with Ang II for 10 min showed, that it was not able to block the Akt signal (Fig. 20*A*), 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[210], the phosphorylation of Akt was even activated, whereas p38 and ERK phosphorylation were inhibited at these high concentrations (Fig. 20*B*-*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[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.

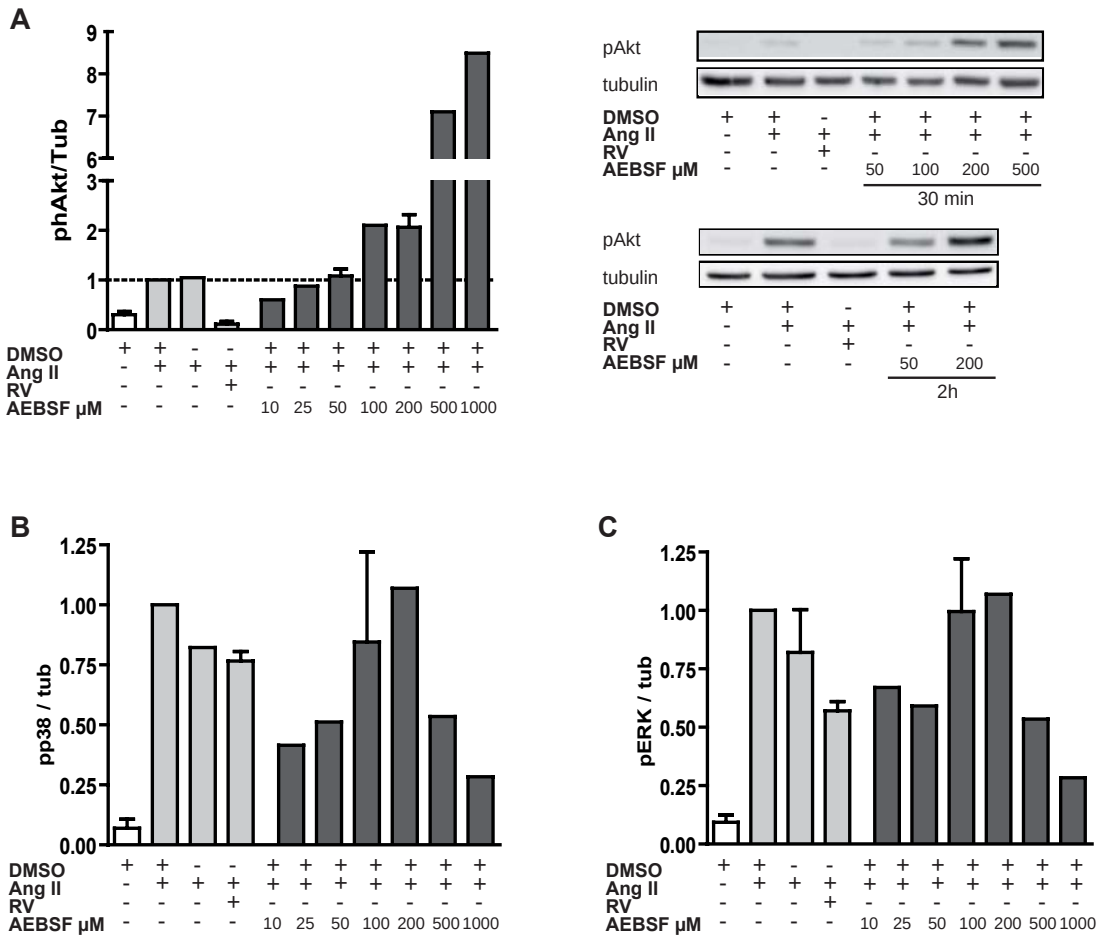

Figure 20: AEBSF has no influence on Ang II-induced phosphorylation of Akt
Quiescent cells were loaded with 10 - 1000 µM AEBSF for 30 min or 2 h and activated with 100 nM Ang II for 10 min followed by western blot analysis using antibodies against pAkt (*A*), pp38 (*B*), pERK1/2 (*C*) and tubulin as 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.[211] We found that Ang II (100 nM, 10 min) induced phosphorylation of Akt was blocked by NAC pre-incubation (Fig. 21*A*) 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. 21*B*). RV had no effect on the phosphorylation of p38 after Ang II stimulation whereas NAC and DPI were able

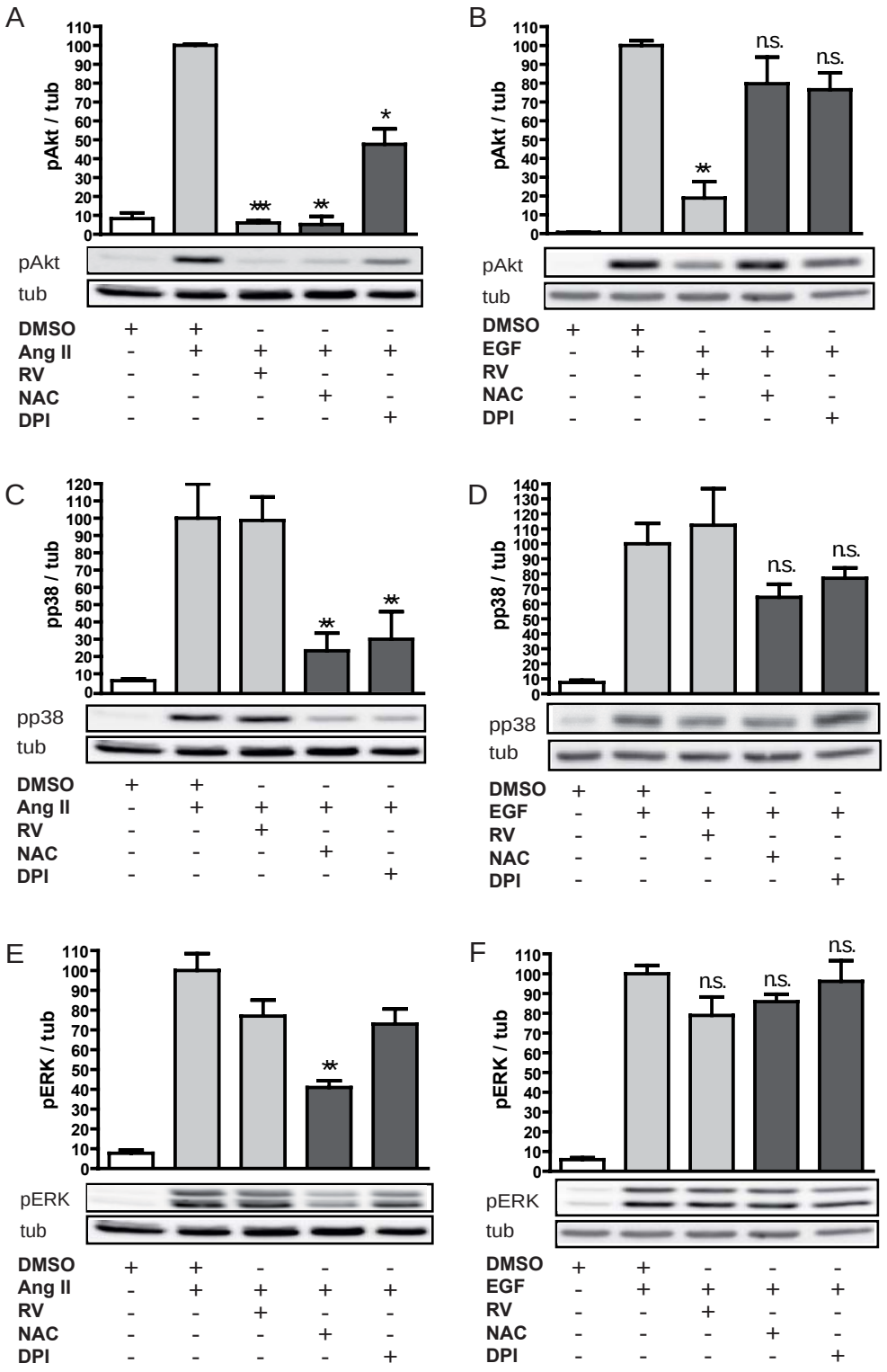

Figure 21: Antioxidants inhibit Ang II-induced phosphorylation of Akt, p38 and ERK
Serum-deprived cells were pretreated with 50 µM RV or DMSO for 30 min, 10 µM DPI for 1 h or 10 mM NAC for 2 h, then stimulated with 100 nM Ang II for 10 min and lysates were subjected to western blot analysis using antibodies 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. 21*C*). In contrast both antioxidants had no effect on p38 phosphorylation after EGF stimulation (Fig. 21*D*). 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. 21*E*). In the EGF stimulated cells no effect of NAC or DPI on the phosphorylation of ERK1/2 could be detected (Fig. 21*F*). 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 ($OH^{\bullet}$) scavengers N-(2-mercaptopropionyl)-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 $OH^{\bullet}$.[212] DMTU is highly diffusible, has a long half-life and high potency in scavenging hydrogen peroxide (H_2O_2) and $OH^{\bullet}$.[213, 214]

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. 22*A*). Pretreatment of cells with 50 – 300 µM MPG for 30 min (Fig. 22*B*) 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 pursuit this effect any longer.

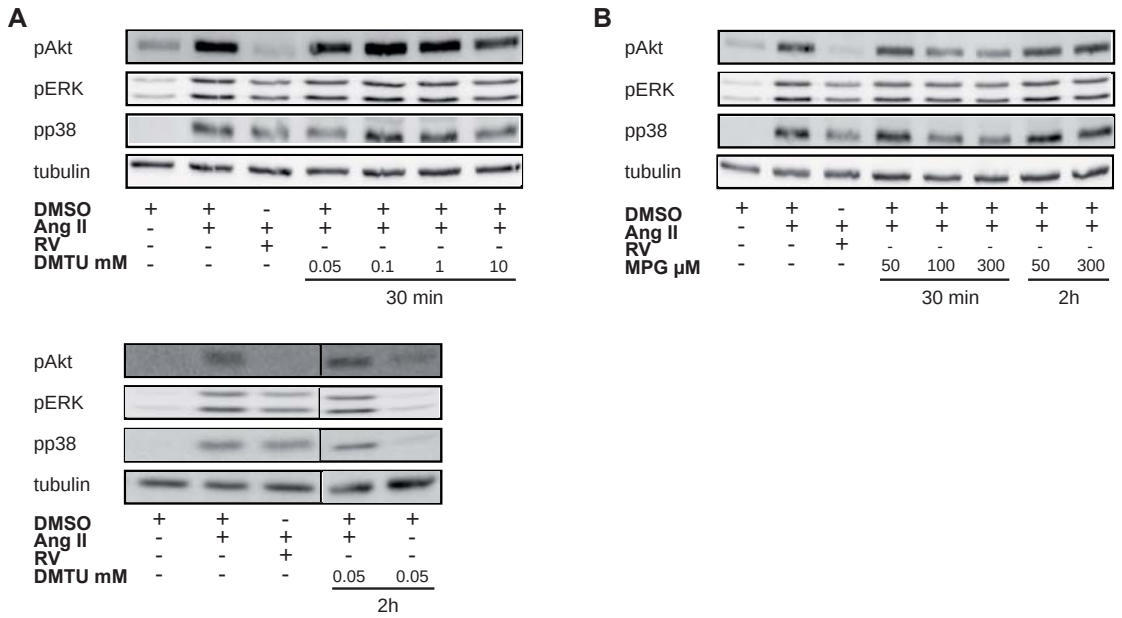

Figure 22: Hydroxyl scavengers cannot inhibit Akt phosphorylation in Ang II-activated VSMC

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 $O_2^{\bullet-}$ to H_2O_2 and catalase is catalysing the reaction of H_2O_2 to water. [161, 162] 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 catalse 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.[215,216] 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 $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ 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.

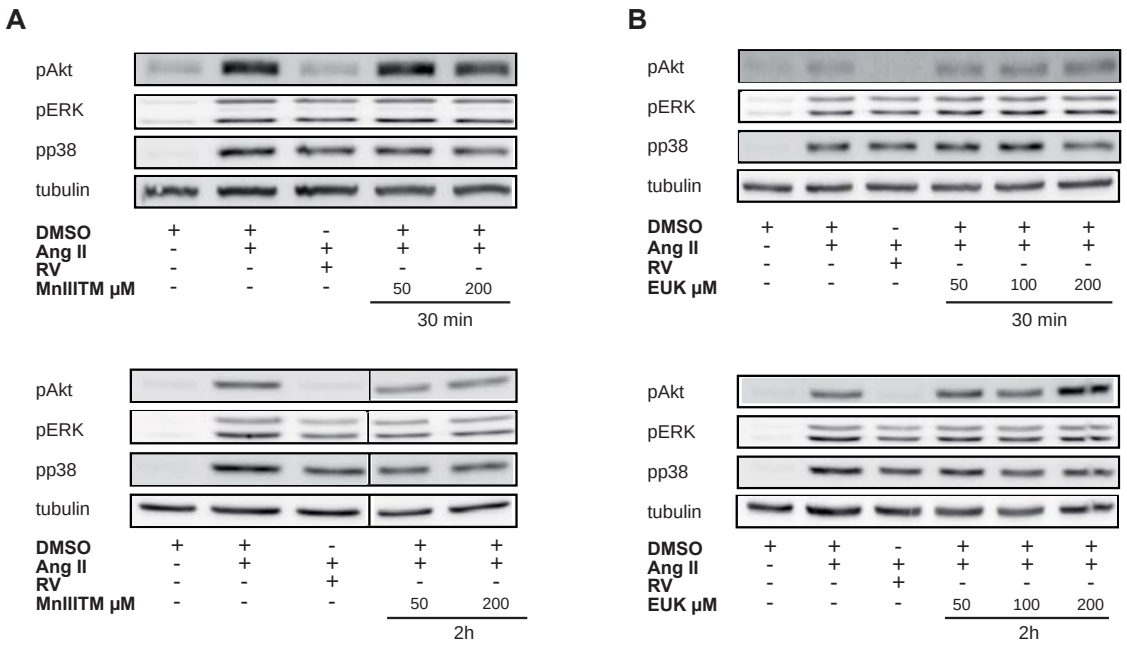

Figure 23: Phosphorylation of Akt is not impeded by SOD and Catalase mimetics in VSMC.
Serum-starved cells were loaded with 50 - 200 µM MnIIITM (*A*) or 50 - 200 µM EUK (*B*) for 30 min or 2 h. Phosphorylation of proteins was detected by western blot analysis after stimulation with 100 nM Ang II for 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[217] and are also target of oxidation leading to inactivation of PTP.[132, 218] Shp2, a PTP, plays a pivotal role in Akt and ERK signaling, negatively influencing Akt and activating ERK[154]. A recent publication indicated that Shp2 is redox-regulated by Ang II[149]. Shp2 was shown to be activated by RV[44], 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 sulfonic acid form of phosphatases[198, 219].

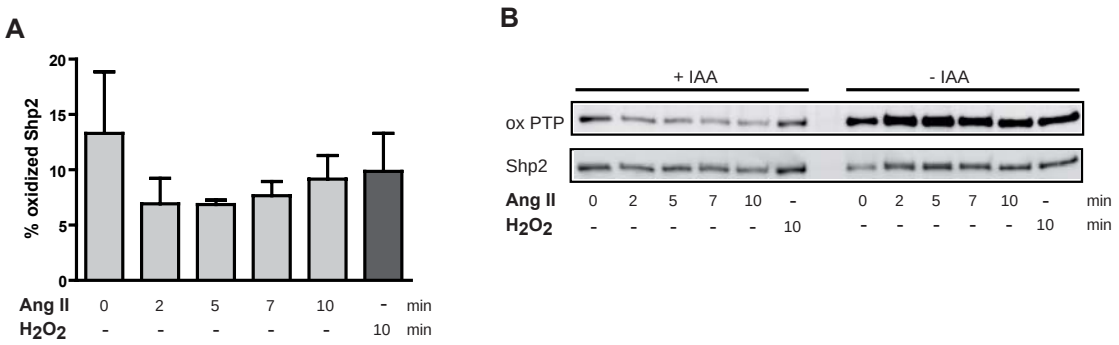

Figure 24: Oxidation of Shp2 after Ang II stimulation
After incubation of quiescent cells with 100 nM Ang II and 3 mM H_2O_2 for indicated times an IP of Shp2 was performed. One half of the samples were treated with IAA to protect reduced (active) forms of Shp2, the other half was used without IAA (positive 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_2O_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_2O_2, 10 min) did not lead to a higher oxidation percentage of oxidized Shp2 compared to the control (Fig. 24*A*-*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. 25*A*) or EGF (Fig. 25*B*) stimulation or RV pretreatment.

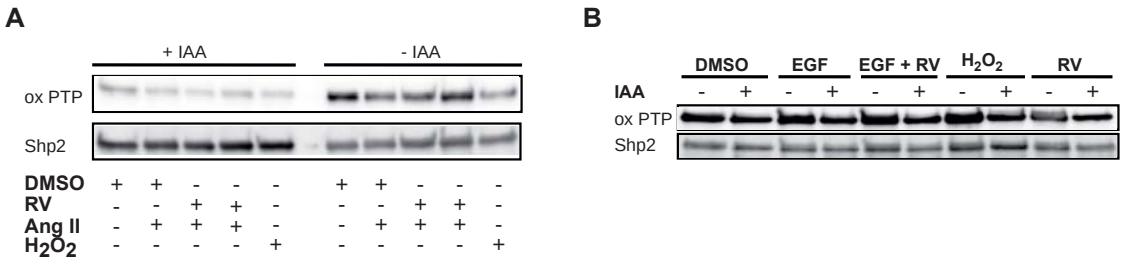

Figure 25: Shp2 oxidation after RV pre-treatment and Ang II or EGF stimulation
Serum-starved cells were stimulated with 100 nM Ang II for 10 min or 100 ng/ml EGF for 5 min after preincubation with 50 µM RV for 30 min or 0.1 % DMSO. 3 mM H_2O_2 stimulation of the cells for 10 min served as positive control. 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.

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. 26*A*), and 3,5-Dihydroxy-4´-methylstilben (R1), methylated at the 4´OH (Fig. 26*B*). 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]

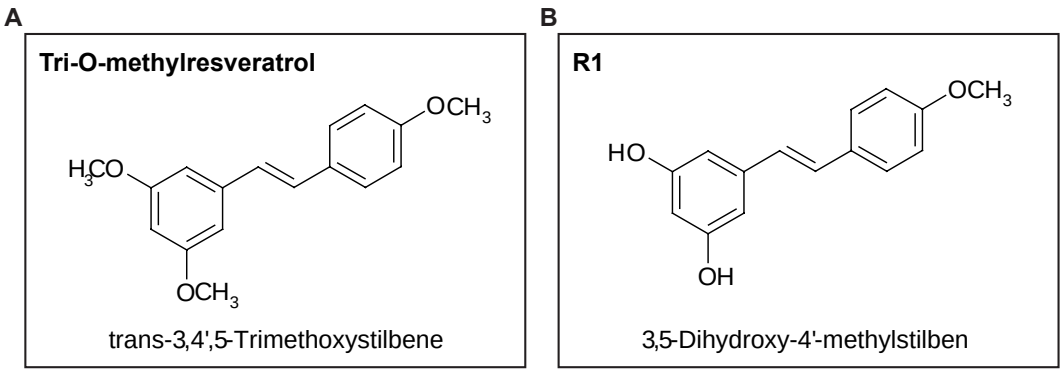

Figure 26: Non-redox active derivatives of RV
In contrast to RV, which possesses three phenolic groups the derivative Tri-O-methylresveratrol (TM-RV) has all three hydroxyl groups methylated (*A*), the derivative 3,5-Dihydroxy-4'-methylstilben (R1) is methylated at the 4´OH (*B*).
Synthesized by the group of Prof. Erker (Department of Pharmaceutical Chemistry, Uni. of Vienna)

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. 27*A*) or EGF (Fig. 27*B*) 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.

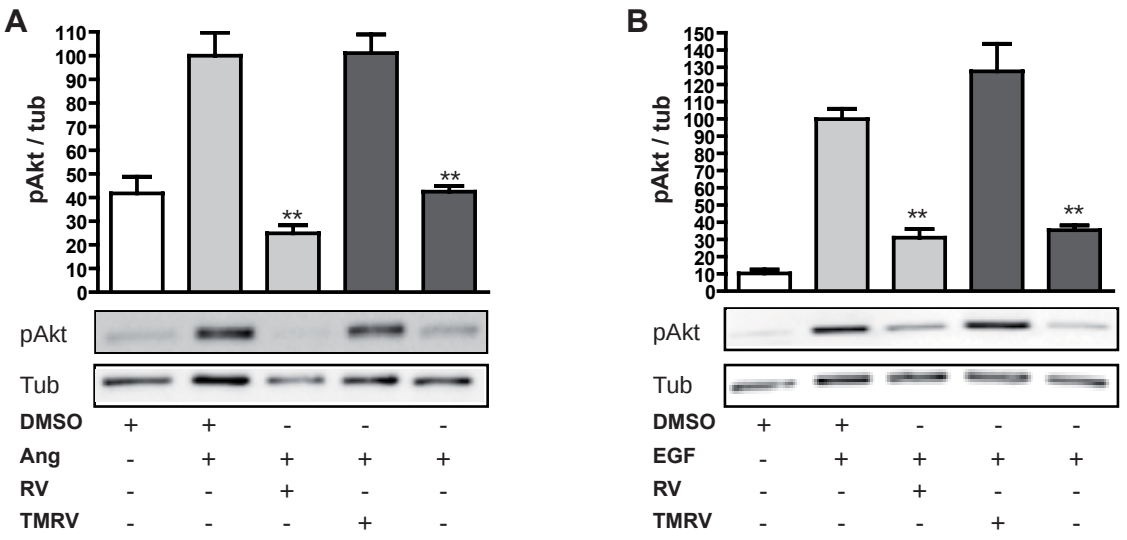

Figure 27: Non-redox active derivatives of RV can inhibit the phosphorylation of Akt after Ang II and EGF stimulation

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^{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[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^{542}, Tyr^{580}) at the C-terminus of Shp2.[131, 140] An induction of phosphorylation of Shp2 after Ang II stimulation was reported recently[149], therefore we checked the phosphorylation status of Shp2 after Ang II and EGF treatment at Tyr^{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^{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

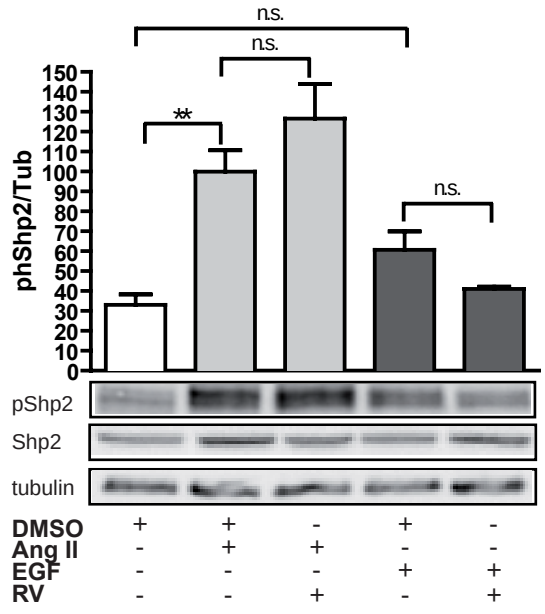

Figure 28: Difference in the phosphorylation of Shp2 upon Ang II or EGF treatment
Shp2 phosphorylation at Tyr^{542} was detected in quiescent cells which were loaded with 50 µM RV or vehicle control for 30 min, then treated with 100 nM Ang II (10 min) or 100 ng/ml EGF (5 min). Following western blotting, 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).

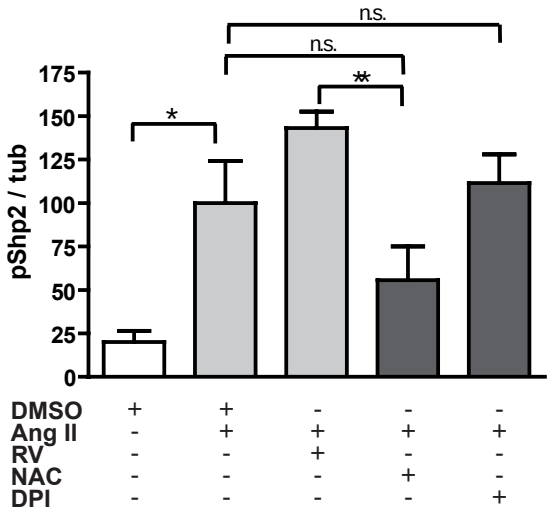

Figure 29: Influence of antioxidants on Shp2 phosphorylation
After pre-treatment of serum-starved cells with 50 µM RV or 0.1 % DMSO for 30 min, incubation with 10 µM DPI (1h) or 10 mM NAC (2h), cells were stimulated with 100 nM Ang II for 10 min. Proteins were subjected to western blot analysis for pShp2 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[220] and PI3K activity after EGF stimulation Shp2 is recruited to the EGF-R[154]. 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.

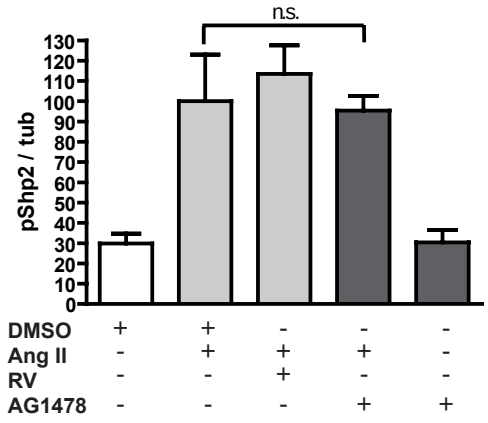

Figure 30: EGFR-kinase activity is not important for the phosphorylation of Shp2 after Ang II stimulation
Phosphorylation of Shp2 was detected by western blotting after incubation of quiescent VSMC with 250 nM AG1478 and 50 µM RV or vehicle control for 30 min, followed by stimulation with Ang II (100 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. 31*A*-*B*) and the phosphorylation of ERK was even blunted to 30 % in the presence of AG1478 (Fig. 31*C*). 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.

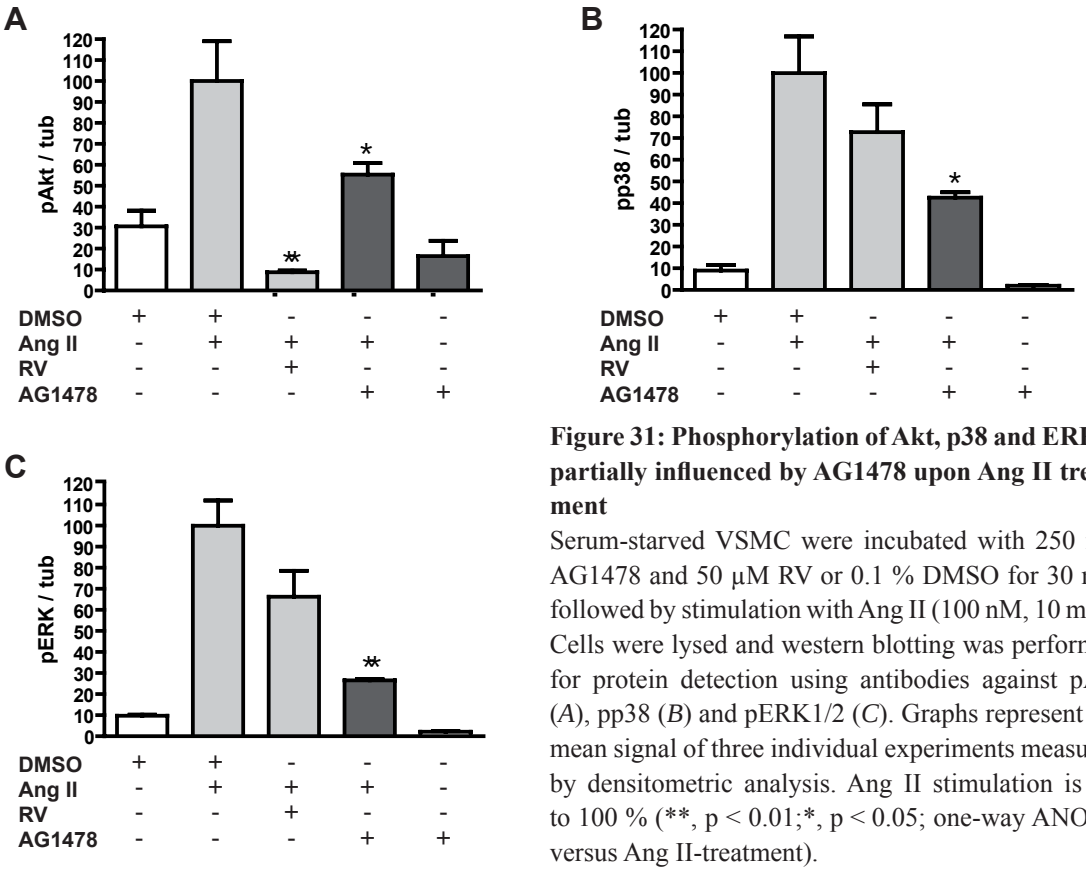

Figure 31: Phosphorylation of Akt, p38 and ERK is partially influenced by AG1478 upon Ang II treatment

Serum-starved VSMC were incubated with 250 nM AG1478 and 50 µM RV or 0.1 % DMSO for 30 min followed by stimulation with Ang II (100 nM, 10 min). Cells were lysed and western blotting was performed for protein detection using antibodies against pAkt (*A*), pp38 (*B*) and pERK1/2 (*C*). Graphs represent the mean signal of three individual experiments measured by densitometric analysis. Ang II stimulation is set to 100 % (**, $p < 0.01$;*, $p < 0.05$; one-way ANOVA versus Ang II-treatment).

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. 32*A*-*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.

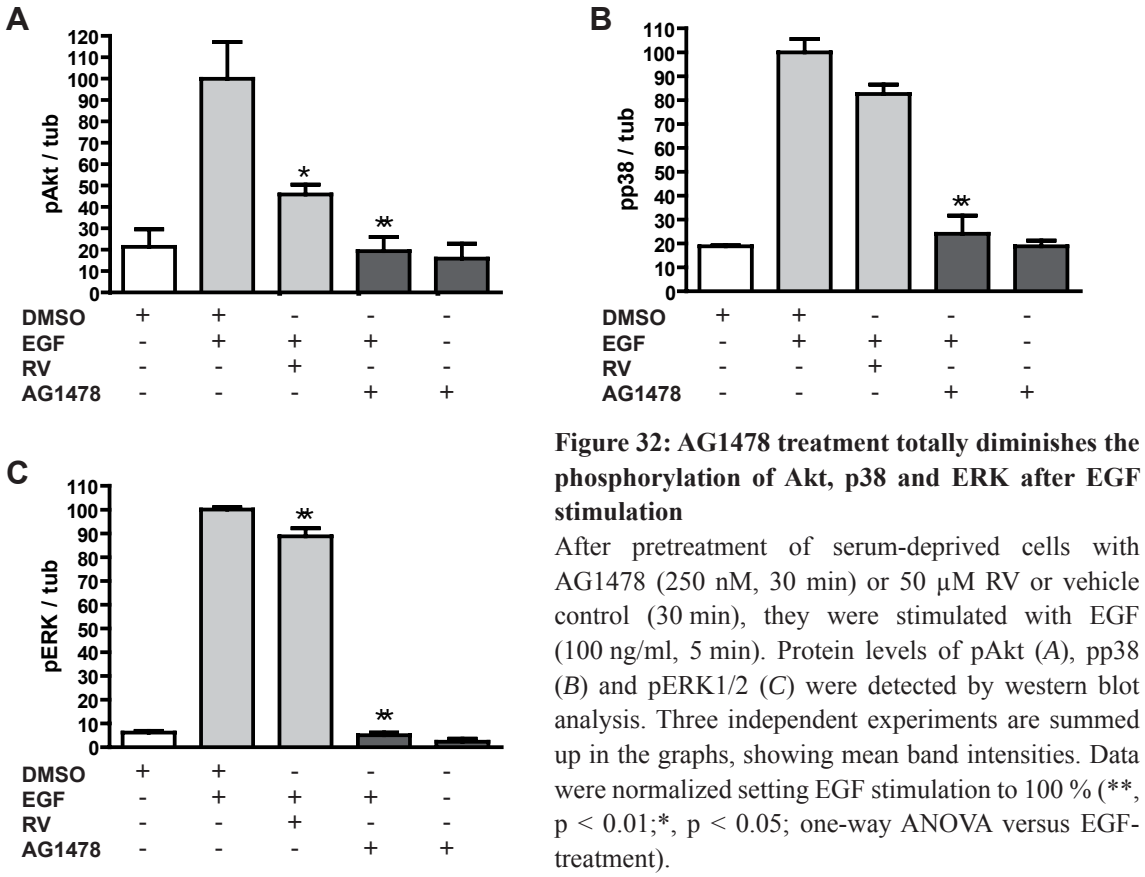

Figure 32: AG1478 treatment totally diminishes the phosphorylation of Akt, p38 and ERK after EGF stimulation

After pretreatment of serum-deprived cells with AG1478 (250 nM, 30 min) or 50 µM RV or vehicle control (30 min), they were stimulated with EGF (100 ng/ml, 5 min). Protein levels of pAkt (*A*), pp38 (*B*) and pERK1/2 (*C*) were detected by western blot analysis. Three independent experiments are summed up in the graphs, showing mean band intensities. Data were normalized setting EGF stimulation to 100 % (**, $p < 0.01$;*, $p < 0.05$; one-way ANOVA versus EGF-treatment).

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.[154] 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.

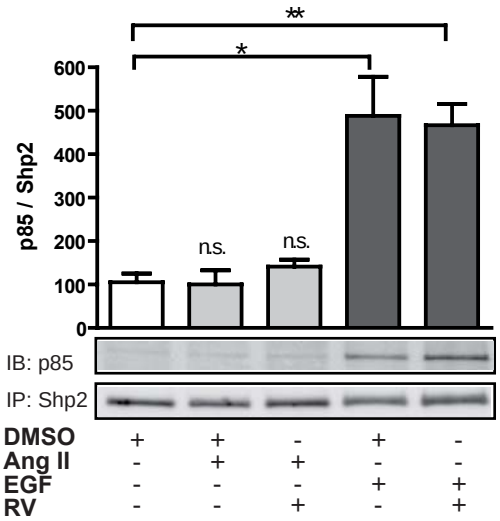

Figure 33: Differences in PI3K recruitment to Shp2 after Ang II and EGF stimulation
After pre-treatment with RV (50 µM, 30 min) or vehicle control quiescent cells were stimulated with 100 nM Ang II (10 min) or 100 ng/ml EGF (5 min). After lysis of the cells an IP of Shp2 was performed and proteins co-precipitated 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. 34*A-B*).

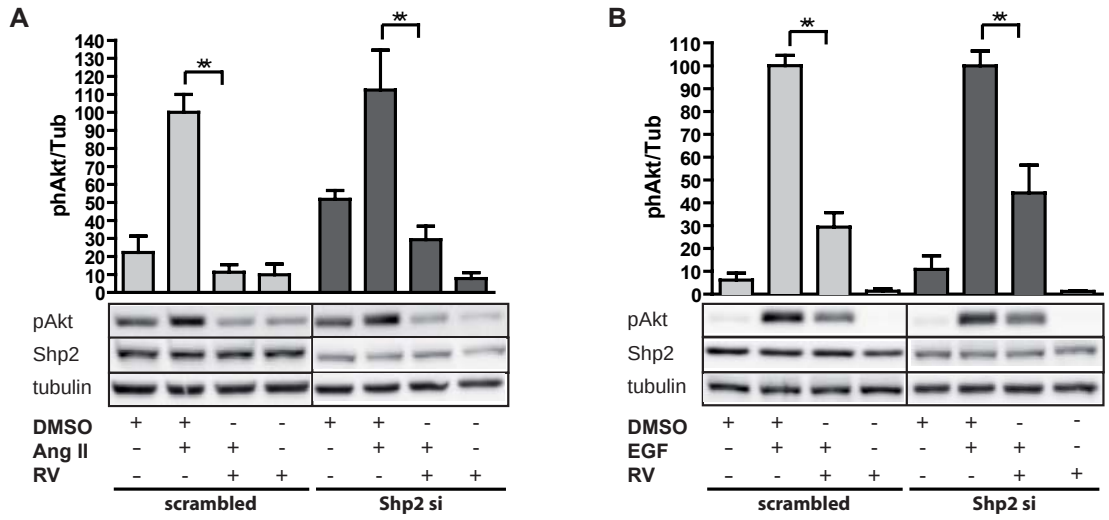

Figure 34: No influence of 50 % Shp2 knock down on Ang II- or EGF-stimulated Akt signaling and RV-mediated inhibition of phospho Akt

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. 35*A-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.

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.

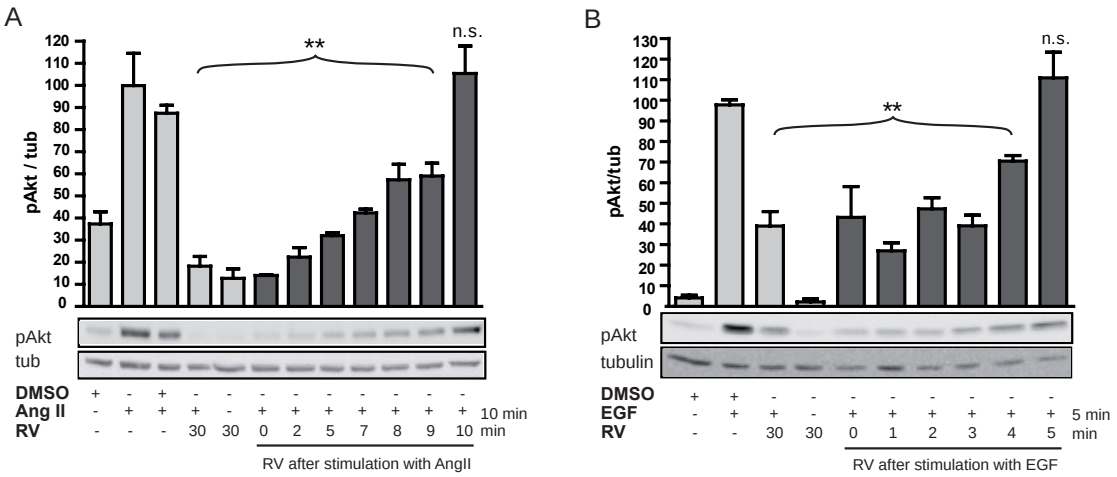

Figure 35: RV treatment after Ang II- and EGF stimulation inhibits phosphorylation of Akt

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[83]. RV was previously shown to inhibit PI3K activity[221], 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[110], 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.

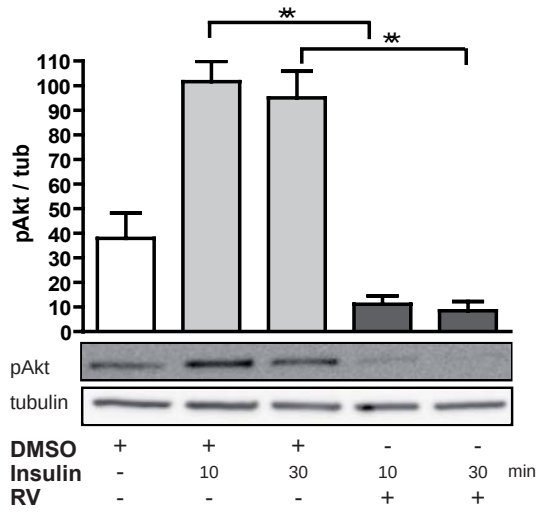

Figure 36: Insulin induced phosphorylation of Akt in VSMC

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.

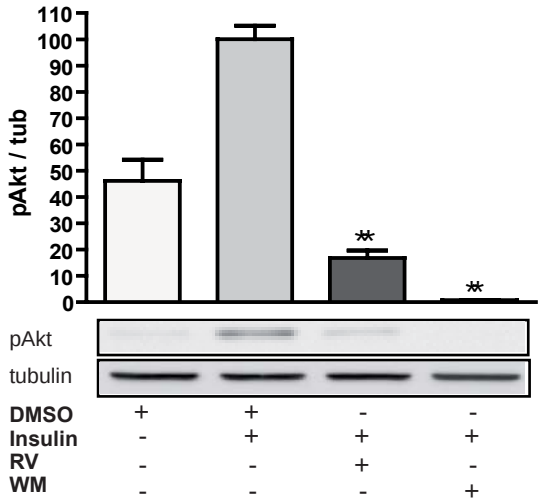

Figure 37: RV and WM block phosphorylation of Akt in VSMC

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.[35] Many different effects of RV were found to be due to activation of sirtuins[33], therefore we applied Sirtinol, a potent SIRT1 inhibitor effective at low concentrations[222] 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. 38*A*-*B*). Referring to these results we exclude an involvement of sirtuins in the repression of Akt phosphorylation by RV in the VSMC.

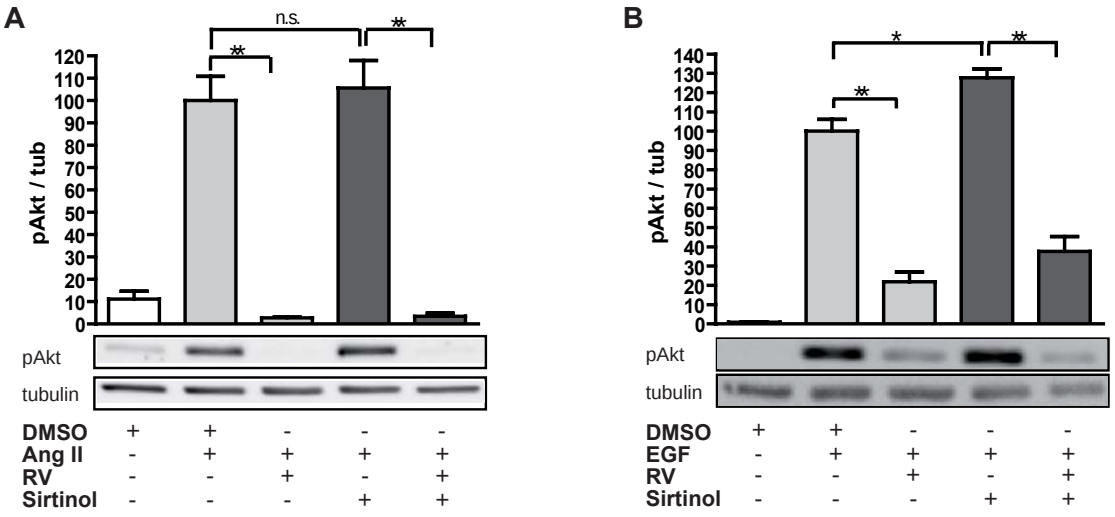

Figure 38: Sirtinol does not influence the RV-mediated inhibition of phosphorylation of Akt in VSMC

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

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 Akt[42-44]. 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 effect[200] 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 cardiomyocytes[45], 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 systems[9, 225]. As EGF-R

phosphorylation was shown to be susceptible for modulation by ROS[226] and the EGF-R was found to also generate H_2O_2 after stimulation of A431 human epidermoid carcinoma cells with EGF[227], we looked if the H_2O_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_2O_2 production is due to an activated EGF-R.[203] These antioxidative properties of RV are thought to include several mechanisms. RV was shown to directly scavenge ROS[21, 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[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[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.[229] Further, Nox4 was found co-localized with the activated EGF-R, indicating a role of both Noxes in Ang II and EGF signaling.[181, 191] In cardiomyocytes Ang II was shown to induce $O_2^{\bullet-}$ in a Nox2 and Rac1 dependent way leading to Akt activation and hypertrophy.[230] RV has already been reported as suppressor of Ang II-induced Nox activity in endothelial cells[48] and it was shown to inhibit LPS-induced Nox1 expression in macrophages leading to inhibition of foam cell formation[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.[189] Moreover, it was also shown before that the EGF-R is able to produce H_2O_2 upon stimulation[203], to co-localize with Nox4 in VSMC when activated[181], and to inactivate PTP1B that is localized at the EGF-R in HAECs[232].

A lack of specific antibodies against Noxes made it impossible to detect Nox protein levels after siRNA treatment of the cells. [180, 233] 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[95], 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[234], and reports showed that Rac1 is needed for the stimulation of NADPH oxidase activity by Ang II[181, 205]. Other groups also showed, that the AT1R and Nox1 are co-localized in caveolae[191], 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.[149] 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[186] 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[208], to the cells led to some reduction of ROS produced after Ang II stimulation, similar to data described in the literature[212, 235, 236] 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[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.[229] However, redox-dependent signaling steps downstream of the EGF-R were suggested in VSMC[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[70], while other groups also reported an inhibition of Ang II-mediated ERK1/2 phosphorylation by DPI[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[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[229, 237]. RV, however, did not interfere with this transactivation, in contrast to NAC[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[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[239]. In human VSMC also a reduction in intracellular hydroxyl radicals after oxLDL incubation was shown when applying 10 mM DMTU[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[212] and preventing H_2O_2-induced left ventricle dysfunction[235]. Also in TNF-α treated myoblasts MPG at a concentration of 10 μM 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 μM 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-β2 induced MMP-2 levels in endothelial cells[242], MnIIITM was shown to similarly reduce IL-β2 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 H_2O_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 considerable 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.

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[243, 244]. Thus methylation could increase metabolic stability and therefore the human oral bioavailability, which would be important when using RV for oral formulations.[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.[220] By dephosphorylation of the PI3K binding site of Gab1 Shp2 can inhibit the Akt phosphorylation in mouse fibroblasts.[154] Also a direct activation of Shp2 by RV was shown[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 .[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.[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 *in vitro* might not be sensitive enough to detect intracellular phosphatases.[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.[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[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[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[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[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.[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[220] and the inhibition of p85 binding to the EGF-R via Gab1[154], other experiments using mouse embryonic fibroblast cells with a deletion at exon 3 ($Shp2^{Ex3-/-}$) - 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.[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.[249] Also a positive effect of Shp2 on Akt/PI3K after stimulation of fibroblasts with interleukin (IL)-3 is reported[250], as well as an activation of Shp2 during IGF-1 induced Akt activation.[251] In addition, in cancer cells, but also in Shp2 $^{Ex3-/-}$ fibroblasts, Shp2 was shown to activate Akt in dependence of EGF and PDGF.[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.[154] 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.[253] 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-R[72], 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 cells[254] and also in preglomerular SMC[255], but that it seems to be dependent on c-src activation in VSMC[256]. In contrast other groups showed a total dependence of ERK1/2 phosphorylation[88] and Akt phosphorylation[257] 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[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[245] and was reported to selectively bind to αVβ3 integrins on the cell surface[259]. A strong dependence of the RV effect on α-estrogen receptor and integrins αVβ3 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.

F REFERENCES

F References

1. Goldschmidt-Clermont, P.J. *et al.* Atherosclerosis 2005: recent discoveries and novel hypotheses. *Circulation* **112**, 3348-3353 (2005).
2. Lusis, A.J. Atherosclerosis. *Nature* **407**, 233-241 (2000).
3. Rader, D.J. & Daugherty, A. Translating molecular discoveries into new therapies for atherosclerosis. *Nature* **451**, 904-913 (2008).
4. Bonomini, F., Tengattini, S., Fabiano, A., Bianchi, R. & Rezzani, R. Atherosclerosis and oxidative stress. *Histology and histopathology* **23**, 381-390 (2008).
5. Dzau, V.J., Braun-Dullaeus, R.C. & Sedding, D.G. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nature medicine* **8**, 1249-1256 (2002).
6. Owens, G.K., Kumar, M.S. & Wamhoff, B.R. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiological reviews* **84**, 767-801 (2004).
7. Ginnan, R., Guikema, B.J., Halligan, K.E., Singer, H.A. & Jourd'heuil, D. Regulation of smooth muscle by inducible nitric oxide synthase and NADPH oxidase in vascular proliferative diseases. *Free radical biology & medicine* **44**, 1232-1245 (2008).
8. Lundberg, M.S. & Crow, M.T. Age-related changes in the signaling and function of vascular smooth muscle cells. *Experimental gerontology* **34**, 549-557 (1999).
9. Baur, J.A. & Sinclair, D.A. Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* **5**, 493-506 (2006).
10. Nonomura, S., Kanagawa, H. & Makimoto, A. [Chemical Constituents of Polygonaceous Plants. I. Studies on the Components of Ko-J O-Kon. (Polygonum Cuspidatum Sieb. Et Zucc.)]. *Yakugaku Zasshi* **83**, 988-990 (1963).
11. Soleas, G.J., Diamandis, E.P. & Goldberg, D.M. Resveratrol: a molecule whose time has come? And gone? *Clinical biochemistry* **30**, 91-113 (1997).
12. Renaud, S. & de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **339**, 1523-1526 (1992).
13. Bertelli, A.A. *et al.* Antiplatelet activity of synthetic and natural resveratrol in red wine. *International journal of tissue reactions* **17**, 1-3 (1995).
14. Wang, Z. *et al.* Effect of resveratrol on platelet aggregation in vivo and in vitro. *Chinese medical journal* **115**, 378-380 (2002).
15. Zini, R., Morin, C., Bertelli, A., Bertelli, A.A. & Tillement, J.P. Effects of resveratrol on the rat brain respiratory chain. *Drugs under experimental and clinical research* **25**, 87-97 (1999).
16. Jang, M. *et al.* Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science (New York, N.Y* **275**, 218-220 (1997).
17. Naderali, E.K., Doyle, P.J. & Williams, G. Resveratrol induces vasorelaxation of mesenteric and uterine arteries from female guinea-pigs. *Clin Sci (Lond)* **98**, 537-543 (2000).
18. Jager, U. & Nguyen-Duong, H. Relaxant effect of trans-resveratrol on isolated porcine coronary arteries. *Arzneimittel-Forschung* **49**, 207-211 (1999).
19. Das, S. *et al.* Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: a potential mechanism for resveratrol preconditioning of the heart. *Vascular pharmacology* **42**, 281-289 (2005).
20. Holvoet, P. Oxidized LDL and coronary heart disease. *Acta cardiologica* **59**, 479-484 (2004).
21. Frankel, E.N., Waterhouse, A.L. & Kinsella, J.E. Inhibition of human LDL oxidation by resveratrol. *Lancet* **341**, 1103-1104 (1993).

22. Whitehead, T.P., Robinson, D., Allaway, S., Syms, J. & Hale, A. Effect of red wine ingestion on the antioxidant capacity of serum. *Clinical chemistry* **41**, 32-35 (1995).
23. Fan, E., Zhang, L., Jiang, S. & Bai, Y. Beneficial effects of resveratrol on atherosclerosis. *Journal of medicinal food* **11**, 610-614 (2008).
24. Fremont, L., Belguendouz, L. & Delpal, S. Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life sciences* **64**, 2511-2521 (1999).
25. Li, Y., Cao, Z. & Zhu, H. Upregulation of endogenous antioxidants and phase 2 enzymes by the red wine polyphenol, resveratrol in cultured aortic smooth muscle cells leads to cytoprotection against oxidative and electrophilic stress. *Pharmacol Res* **53**, 6-15 (2006).
26. Cao, Z. & Li, Y. Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury. *European journal of pharmacology* **489**, 39-48 (2004).
27. Stivala, L.A. *et al.* Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *The Journal of biological chemistry* **276**, 22586-22594 (2001).
28. Murias, M. *et al.* Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship. *Biochemical pharmacology* **69**, 903-912 (2005).
29. Hung, L.M. *et al.* The protective effect of resveratrols on ischaemia-reperfusion injuries of rat hearts is correlated with antioxidant efficacy. *British journal of pharmacology* **135**, 1627-1633 (2002).
30. Udenigwe, C.C., Ramprasath, V.R., Aluko, R.E. & Jones, P.J. Potential of resveratrol in anticancer and anti-inflammatory therapy. *Nutrition reviews* **66**, 445-454 (2008).
31. Kundu, J.K. & Surh, Y.J. Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. *Cancer letters* **269**, 243-261 (2008).
32. Khan, N., Afaq, F. & Mukhtar, H. Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxidants & redox signaling* **10**, 475-510 (2008).
33. Denu, J.M. The Sir 2 family of protein deacetylases. *Current opinion in chemical biology* **9**, 431-440 (2005).
34. Glozak, M.A., Sengupta, N., Zhang, X. & Seto, E. Acetylation and deacetylation of non-histone proteins. *Gene* **363**, 15-23 (2005).
35. Howitz, K.T. *et al.* Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* **425**, 191-196 (2003).
36. Wood, J.G. *et al.* Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* **430**, 686-689 (2004).
37. Valenzano, D.R. *et al.* Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. *Curr Biol* **16**, 296-300 (2006).
38. McBurney, M.W. *et al.* The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. *Molecular and cellular biology* **23**, 38-54 (2003).
39. Kaeberlein, M. *et al.* Substrate-specific activation of sirtuins by resveratrol. *The Journal of biological chemistry* **280**, 17038-17045 (2005).
40. Cucciolla, V. *et al.* Resveratrol: from basic science to the clinic. *Cell cycle (Georgetown, Tex* **6**, 2495-2510 (2007).
41. Ohtsu, H., Frank, G.D., Utsunomiya, H. & Eguchi, S. Redox-dependent protein kinase regulation by angiotensin II: mechanistic insights and its pathophysiology. *Antioxidants & redox signaling* **7**, 1315-1326 (2005).

42. Haider, U.G., Sorescu, D., Griendling, K.K., Vollmar, A.M. & Dirsch, V.M. Resveratrol suppresses angiotensin II-induced Akt/protein kinase B and p70 S6 kinase phosphorylation and subsequent hypertrophy in rat aortic smooth muscle cells. *Mol Pharmacol* **62**, 772-777 (2002).
43. Haider, U.G., Sorescu, D., Griendling, K.K., Vollmar, A.M. & Dirsch, V.M. Resveratrol increases serine15-phosphorylated but transcriptionally impaired p53 and induces a reversible DNA replication block in serum-activated vascular smooth muscle cells. *Mol Pharmacol* **63**, 925-932 (2003).
44. Haider, U.G. *et al.* Resveratrol inhibits angiotensin II- and epidermal growth factor-mediated Akt activation: role of Gab1 and Shp2. *Mol Pharmacol* **68**, 41-48 (2005).
45. Cheng, T.H. *et al.* Inhibitory effect of resveratrol on angiotensin II-induced cardiomyocyte hypertrophy. *Naunyn-Schmiedeberg's archives of pharmacology* **369**, 239-244 (2004).
46. Liu, Z. *et al.* Effects of trans-resveratrol on hypertension-induced cardiac hypertrophy using the partially nephrectomized rat model. *Clinical and experimental pharmacology & physiology* **32**, 1049-1054 (2005).
47. Olson, E.R., Naugle, J.E., Zhang, X., Bomser, J.A. & Meszaros, J.G. Inhibition of cardiac fibroblast proliferation and myofibroblast differentiation by resveratrol. *American journal of physiology* **288**, H1131-1138 (2005).
48. Chow, S.E., Hshu, Y.C., Wang, J.S. & Chen, J.K. Resveratrol attenuates oxLDL-stimulated NADPH oxidase activity and protects endothelial cells from oxidative functional damages. *J Appl Physiol* **102**, 1520-1527 (2007).
49. Inanaga, K. *et al.* Resveratrol attenuates angiotensin II-induced interleukin-6 expression and perivascular fibrosis. *Hypertens Res* (2009).
50. Marier, J.F. *et al.* Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. *The Journal of pharmacology and experimental therapeutics* **302**, 369-373 (2002).
51. Asensi, M. *et al.* Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free radical biology & medicine* **33**, 387-398 (2002).
52. Saiko, P., Szakmary, A., Jaeger, W. & Szekeres, T. Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? *Mutation research* **658**, 68-94 (2008).
53. Walle, T., Hsieh, F., DeLegge, M.H., Oatis, J.E., Jr. & Walle, U.K. High absorption but very low bioavailability of oral resveratrol in humans. *Drug metabolism and disposition: the biological fate of chemicals* **32**, 1377-1382 (2004).
54. Geisterfer, A.A., Peach, M.J. & Owens, G.K. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circulation research* **62**, 749-756 (1988).
55. Das, D.K., Maulik, N. & Engelman, R.M. Redox regulation of angiotensin II signaling in the heart. *Journal of cellular and molecular medicine* **8**, 144-152 (2004).
56. Marchesi, C., Paradis, P. & Schiffrin, E.L. Role of the renin-angiotensin system in vascular inflammation. *Trends in pharmacological sciences* **29**, 367-374 (2008).
57. Touyz, R.M. Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease. *Braz J Med Biol Res* **37**, 1263-1273 (2004).
58. Touyz, R.M. & Schiffrin, E.L. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacological reviews* **52**, 639-672 (2000).
59. Kim, S. & Iwao, H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacological reviews* **52**, 11-34 (2000).
60. Mehta, P.K. & Griendling, K.K. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* **292**, C82-97 (2007).

61. Lombardi, D. *et al.* Salt-sensitive hypertension develops after short-term exposure to Angiotensin II. *Hypertension* **33**, 1013-1019 (1999).
62. Zhang, G.X., Lu, X.M., Kimura, S. & Nishiyama, A. Role of mitochondria in angiotensin II-induced reactive oxygen species and mitogen-activated protein kinase activation. *Cardiovascular research* **76**, 204-212 (2007).
63. Allen, A.M., Zhuo, J. & Mendelsohn, F.A. Localization and function of angiotensin AT1 receptors. *American journal of hypertension* **13**, 31S-38S (2000).
64. Shah, B.H. & Catt, K.J. A central role of EGF receptor transactivation in angiotensin II -induced cardiac hypertrophy. *Trends in pharmacological sciences* **24**, 239-244 (2003).
65. Inagami, T. & Eguchi, S. Angiotensin II-mediated vascular smooth muscle cell growth signaling. *Braz J Med Biol Res* **33**, 619-624 (2000).
66. Lassegue, B. *et al.* Angiotensin II down-regulates the vascular smooth muscle AT1 receptor by transcriptional and post-transcriptional mechanisms: evidence for homologous and heterologous regulation. *Molecular pharmacology* **48**, 601-609 (1995).
67. Gunther, S., Gimbrone, M.A., Jr. & Alexander, R.W. Regulation by angiotensin II of its receptors in resistance blood vessels. *Nature* **287**, 230-232 (1980).
68. Griendling, K.K., Delafontaine, P., Rittenhouse, S.E., Gimbrone, M.A., Jr. & Alexander, R.W. Correlation of receptor sequestration with sustained diacylglycerol accumulation in angiotensin II-stimulated cultured vascular smooth muscle cells. *The Journal of biological chemistry* **262**, 14555-14562 (1987).
69. Griendling, K.K., Sorescu, D. & Ushio-Fukai, M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circulation research* **86**, 494-501 (2000).
70. Frank, G.D., Eguchi, S., Inagami, T. & Motley, E.D. N-acetylcysteine inhibits angiotensin II-mediated activation of extracellular signal-regulated kinase and epidermal growth factor receptor. *Biochem Biophys Res Commun* **280**, 1116-1119 (2001).
71. Touyz, R.M. *et al.* Redox-dependent MAP kinase signaling by Ang II in vascular smooth muscle cells: role of receptor tyrosine kinase transactivation. *Canadian journal of physiology and pharmacology* **81**, 159-167 (2003).
72. Higuchi, S. *et al.* Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology. *Clin Sci (Lond)* **112**, 417-428 (2007).
73. Kyaw, M., Yoshizumi, M., Tsuchiya, K., Kirima, K. & Tamaki, T. Antioxidants inhibit JNK and p38 MAPK activation but not ERK 1/2 activation by angiotensin II in rat aortic smooth muscle cells. *Hypertens Res* **24**, 251-261 (2001).
74. Ichijo, H. *et al.* Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science (New York, N.Y* **275**, 90-94 (1997).
75. Eguchi, S., Dempsey, P.J., Frank, G.D., Motley, E.D. & Inagami, T. Activation of MAPKs by angiotensin II in vascular smooth muscle cells. Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. *The Journal of biological chemistry* **276**, 7957-7962 (2001).
76. Dreux, A.C., Lamb, D.J., Modjtahedi, H. & Ferns, G.A. The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. *Atherosclerosis* **186**, 38-53 (2006).
77. Burgess, A.W. Epidermal growth factor and transforming growth factor alpha. *British medical bulletin* **45**, 401-424 (1989).
78. Mitsumata, M., Gamou, S., Shimizu, N. & Yoshida, Y. Response of atherosclerotic intimal smooth muscle cells to epidermal growth factor in vitro. *Arterioscler Thromb* **14**, 1364-1371 (1994).
79. Yamanaka, Y. *et al.* EGF family ligand-dependent phenotypic modulation of smooth muscle cells through EGF receptor. *Biochem Biophys Res Commun* **281**, 373-377 (2001).

80. Citri, A. & Yarden, Y. EGF-ERBB signalling: towards the systems level. *Nature reviews* **7**, 505-516 (2006).

81. Tice, D.A., Biscardi, J.S., Nickles, A.L. & Parsons, S.J. Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 1415-1420 (1999).

82. Jorissen, R.N. *et al.* Epidermal growth factor receptor: mechanisms of activation and signalling. *Experimental cell research* **284**, 31-53 (2003).

83. Rodrigues, G.A., Falasca, M., Zhang, Z., Ong, S.H. & Schlessinger, J. A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. *Molecular and cellular biology* **20**, 1448-1459 (2000).

84. Wiley, H.S. Trafficking of the ErbB receptors and its influence on signaling. *Experimental cell research* **284**, 78-88 (2003).

85. Anderson, R.G. The caveolae membrane system. *Annual review of biochemistry* **67**, 199-225 (1998).

86. Waugh, M.G., Lawson, D. & Hsuan, J.J. Epidermal growth factor receptor activation is localized within low-buoyant density, non-caveolar membrane domains. *The Biochemical journal* **337 (Pt3)**, 591-597 (1999).

87. Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211-225 (2000).

88. Eguchi, S. *et al.* Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *The Journal of biological chemistry* **273**, 8890-8896 (1998).

89. Rao, G.N. Hydrogen peroxide induces complex formation of SHC-Grb2-SOS with receptor tyrosine kinase and activates Ras and extracellular signal-regulated protein kinases group of mitogen-activated protein kinases. *Oncogene* **13**, 713-719 (1996).

90. Iwasaki, H., Eguchi, S., Ueno, H., Marumo, F. & Hirata, Y. Endothelin-mediated vascular growth requires p42/p44 mitogen-activated protein kinase and p70 S6 kinase cascades via transactivation of epidermal growth factor receptor. *Endocrinology* **140**, 4659-4668 (1999).

91. Kalmes, A., Vesti, B.R., Daum, G., Abraham, J.A. & Clowes, A.W. Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor. *Circulation research* **87**, 92-98 (2000).

92. Suc, I. *et al.* Activation of EGF receptor by oxidized LDL. *Faseb J* **12**, 665-671 (1998).

93. Iwasaki, H., Eguchi, S., Ueno, H., Marumo, F. & Hirata, Y. Mechanical stretch stimulates growth of vascular smooth muscle cells via epidermal growth factor receptor. *American journal of physiology* **278**, H521-529 (2000).

94. Frank, G.D. & Eguchi, S. Activation of tyrosine kinases by reactive oxygen species in vascular smooth muscle cells: significance and involvement of EGF receptor transactivation by angiotensin II. *Antioxidants & redox signaling* **5**, 771-780 (2003).

95. Ushio-Fukai, M. *et al.* Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **21**, 489-495 (2001).

96. Ushio-Fukai, M. *et al.* Cholesterol depletion inhibits epidermal growth factor receptor transactivation by angiotensin II in vascular smooth muscle cells: role of cholesterol-rich microdomains and focal adhesions in angiotensin II signaling. *The Journal of biological chemistry* **276**, 48269-48275 (2001).

97. Ohtsu, H. *et al.* ADAM17 mediates epidermal growth factor receptor transactivation and vascular smooth muscle cell hypertrophy induced by angiotensin II. *Arterioscler Thromb Vasc Biol* **26**, e133-137 (2006).

98. Suzuki, H., Motley, E.D., Frank, G.D., Utsunomiya, H. & Eguchi, S. Recent progress in signal transduction research of the angiotensin II type-1 receptor: protein kinases, vascular dysfunction and structural requirement. *Current medicinal chemistry* **3**, 305-322 (2005).

99. Sowers, J.R. Insulin resistance and hypertension. *American journal of physiology* **286**, H1597-1602 (2004).

100. Taniguchi, C.M., Emanuelli, B. & Kahn, C.R. Critical nodes in signalling pathways: insights into insulin action. *Nature reviews* **7**, 85-96 (2006).

101. LeRoith, D., Werner, H., Beitner-Johnson, D. & Roberts, C.T., Jr. Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocrine reviews* **16**, 143-163 (1995).

102. Olivares-Reyes, J.A., Arellano-Plancarte, A. & Castillo-Hernandez, J.R. Angiotensin II and the development of insulin resistance: Implications for diabetes. *Molecular and cellular endocrinology* **302**, 128-139 (2009).

103. Folli, F., Kahn, C.R., Hansen, H., Bouchie, J.L. & Feener, E.P. Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels. A potential role for serine phosphorylation in insulin/angiotensin II crosstalk. *The Journal of clinical investigation* **100**, 2158-2169 (1997).

104. Taniyama, Y., Hitomi, H., Shah, A., Alexander, R.W. & Griendling, K.K. Mechanisms of reactive oxygen species-dependent downregulation of insulin receptor substrate-1 by angiotensin II. *Arterioscler Thromb Vasc Biol* **25**, 1142-1147 (2005).

105. Marone, R., Cmiljanovic, V., Giese, B. & Wymann, M.P. Targeting phosphoinositide 3-kinase: moving towards therapy. *Biochimica et biophysica acta* **1784**, 159-185 (2008).

106. Woodgett, J.R. Recent advances in the protein kinase B signaling pathway. *Current opinion in cell biology* **17**, 150-157 (2005).

107. Oudit, G.Y. *et al.* The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *Journal of molecular and cellular cardiology* **37**, 449-471 (2004).

108. Dorn, G.W., 2nd & Force, T. Protein kinase cascades in the regulation of cardiac hypertrophy. *The Journal of clinical investigation* **115**, 527-537 (2005).

109. Manning, B.D. & Cantley, L.C. AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274 (2007).

110. Sale, E.M. & Sale, G.J. Protein kinase B: signalling roles and therapeutic targeting. *Cell Mol Life Sci* **65**, 113-127 (2008).

111. Jacinto, E. *et al.* SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**, 125-137 (2006).

112. Sarbassov, D.D., Ali, S.M. & Sabatini, D.M. Growing roles for the mTOR pathway. *Current opinion in cell biology* **17**, 596-603 (2005).

113. Bozulic, L. & Hemmings, B.A. PIKKing on PKB: regulation of PKB activity by phosphorylation. *Current opinion in cell biology* **21**, 256-261 (2009).

114. Cohen, P. & Frame, S. The renaissance of GSK3. *Nature reviews* **2**, 769-776 (2001).

115. Maurer, U., Charvet, C., Wagman, A.S., Dejardin, E. & Green, D.R. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Molecular cell* **21**, 749-760 (2006).

116. Datta, S.R. *et al.* Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-241 (1997).

117. Burgering, B.M. & Kops, G.J. Cell cycle and death control: long live Forkheads. *Trends in biochemical sciences* **27**, 352-360 (2002).

118. Yu, P.J. *et al.* Vascular injury and modulation of MAPKs: a targeted approach to therapy of restenosis. *Cellular signalling* **19**, 1359-1371 (2007).

119.Johnson, G.L. & Lapadat, R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science (New York, N.Y* **298**, 1911-1912 (2002).

120.Muto, A. *et al.* Smooth muscle cell signal transduction: implications of vascular biology for vascular surgeons. *J Vasc Surg* **45 Suppl A**, A15-24 (2007).

121.Dance, M., Montagner, A., Salles, J.P., Yart, A. & Raynal, P. The molecular functions of Shp2 in the Ras/Mitogen-activated protein kinase (ERK1/2) pathway. *Cellular signalling* **20**, 453-459 (2008).

122.Muslin, A.J. MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets. *Clin Sci (Lond)* **115**, 203-218 (2008).

123.Takahashi, E. & Berk, B.C. MAP kinases and vascular smooth muscle function. *Acta physiologica Scandinavica* **164**, 611-621 (1998).

124.Hu, Y., Cheng, L., Hochleitner, B.W. & Xu, Q. Activation of mitogen-activated protein kinases (ERK/JNK) and AP-1 transcription factor in rat carotid arteries after balloon injury. *Arterioscler Thromb Vasc Biol* **17**, 2808-2816 (1997).

125.Lai, K. *et al.* Mitogen-activated protein kinase phosphatase-1 in rat arterial smooth muscle cell proliferation. *The Journal of clinical investigation* **98**, 1560-1567 (1996).

126.Zhao, M. *et al.* Activation of the p38 MAP kinase pathway is required for foam cell formation from macrophages exposed to oxidized LDL. *Apmis* **110**, 458-468 (2002).

127.Ostman, A., Hellberg, C. & Bohmer, F.D. Protein-tyrosine phosphatases and cancer. *Nat Rev Cancer* **6**, 307-320 (2006).

128.Tonks, N.K. Protein tyrosine phosphatases: from genes, to function, to disease. *Nature reviews* **7**, 833-846 (2006).

129.Monteiro, H.P., Arai, R.J. & Travassos, L.R. Protein tyrosine phosphorylation and protein tyrosine nitration in redox signaling. *Antioxidants & redox signaling* **10**, 843-889 (2008).

130.Chiarugi, P. & Buricchi, F. Protein tyrosine phosphorylation and reversible oxidation: two cross-talking posttranslation modifications. *Antioxidants & redox signaling* **9**, 1-24 (2007).

131.den Hertog, J., Ostman, A. & Bohmer, F.D. Protein tyrosine phosphatases: regulatory mechanisms. *The FEBS journal* **275**, 831-847 (2008).

132.Tonks, N.K. Redox redux: revisiting PTPs and the control of cell signaling. *Cell* **121**, 667-670 (2005).

133.Meng, T.C., Fukada, T. & Tonks, N.K. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Molecular cell* **9**, 387-399 (2002).

134.Chiarugi, P. Reactive oxygen species as mediators of cell adhesion. *The Italian journal of biochemistry* **52**, 28-32 (2003).

135.Chen, C.Y., Willard, D. & Rudolph, J. Redox Regulation of SH2-Domain-Containing Protein Tyrosine Phosphatases by Two Backdoor Cysteines. *Biochemistry* (2009).

136.Luttrell, D.K. & Luttrell, L.M. Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. *Oncogene* **23**, 7969-7978 (2004).

137.Kodama, H. *et al.* Selective involvement of p130Cas/Crk/Pyk2/c-Src in endothelin-1-induced JNK activation. *Hypertension* **41**, 1372-1379 (2003).

138.Chiarugi, P. & Cirri, P. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. *Trends in biochemical sciences* **28**, 509-514 (2003).

139.Shen, K. Analyzing protein tyrosine phosphatases by phosphotyrosine analog integration. *Methods* **42**, 234-242 (2007).

140.Poole, A.W. & Jones, M.L. A SHPing tale: perspectives on the regulation of SHP-1 and SHP-2 tyrosine phosphatases by the C-terminal tail. *Cellular signalling* **17**, 1323-1332 (2005).

141.Kappert, K., Peters, K.G., Bohmer, F.D. & Ostman, A. Tyrosine phosphatases in vessel wall signaling. *Cardiovascular research* **65**, 587-598 (2005).

142.Chang, Y., Zhuang, D., Zhang, C. & Hassid, A. Increase of PTP levels in vascular injury and in cultured aortic smooth muscle cells treated with specific growth factors. *American journal of physiology* **287**, H2201-2208 (2004).

143.Neel, B.G., Gu, H. & Pao, L. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends in biochemical sciences* **28**, 284-293 (2003).

144.Strack, V. *et al.* The Protein-tyrosine-phosphatase SHP2 is phosphorylated on serine residues 576 and 591 by protein kinase C isoforms alpha, beta 1, beta 2, and eta. *Biochemistry* **41**, 603-608 (2002).

145.Araki, T., Nawa, H. & Neel, B.G. Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors. *The Journal of biological chemistry* **278**, 41677-41684 (2003).

146.Bennett, A.M., Tang, T.L., Sugimoto, S., Walsh, C.T. & Neel, B.G. Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor beta to Ras. *Proc Natl Acad Sci U S A* **91**, 7335-7339 (1994).

147.Vogel, W. & Ullrich, A. Multiple in vivo phosphorylated tyrosine phosphatase SHP-2 engages binding to Grb2 via tyrosine 584. *Cell Growth Differ* **7**, 1589-1597 (1996).

148.Weibrecht, I. *et al.* Oxidation sensitivity of the catalytic cysteine of the protein-tyrosine phosphatases SHP-1 and SHP-2. *Free radical biology & medicine* **43**, 100-110 (2007).

149.Tabet, F. *et al.* Redox-sensitive signaling by angiotensin II involves oxidative inactivation and blunted phosphorylation of protein tyrosine phosphatase SHP-2 in vascular smooth muscle cells from SHR. *Circulation research* **103**, 149-158 (2008).

150.Qu, C.K., Yu, W.M., Azzarelli, B. & Feng, G.S. Genetic evidence that Shp-2 tyrosine phosphatase is a signal enhancer of the epidermal growth factor receptor in mammals. *Proc Natl Acad Sci U S A* **96**, 8528-8533 (1999).

151.Qu, C.K. The SHP-2 tyrosine phosphatase: signaling mechanisms and biological functions. *Cell research* **10**, 279-288 (2000).

152.Agazie, Y.M. & Hayman, M.J. Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling. *Molecular and cellular biology* **23**, 7875-7886 (2003).

153.Montagner, A. *et al.* A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation. *The Journal of biological chemistry* **280**, 5350-5360 (2005).

154.Zhang, S.Q. *et al.* Receptor-specific regulation of phosphatidylinositol 3'-kinase activation by the protein tyrosine phosphatase Shp2. *Molecular and cellular biology* **22**, 4062-4072 (2002).

155.Seta, K. & Sadoshima, J. Phosphorylation of tyrosine 319 of the angiotensin II type 1 receptor mediates angiotensin II-induced trans-activation of the epidermal growth factor receptor. *The Journal of biological chemistry* **278**, 9019-9026 (2003).

156.Sampaio, W.O., Henrique de Castro, C., Santos, R.A., Schiffrin, E.L. & Touyz, R.M. Angiotensin-(1-7) counterregulates angiotensin II signaling in human endothelial cells. *Hypertension* **50**, 1093-1098 (2007).

157.Paravicini, T.M. & Touyz, R.M. Redox signaling in hypertension. *Cardiovascular research* **71**, 247-258 (2006).

158.D'Autreaux, B. & Toledano, M.B. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nature reviews* **8**, 813-824 (2007).

159.Valko, M. *et al.* Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology* **39**, 44-84 (2007).

160.Lee, M.Y. & Griendling, K.K. Redox signaling, vascular function, and hypertension. *Antioxidants & redox signaling* **10**, 1045-1059 (2008).

161.Ardanaz, N. & Pagano, P.J. Hydrogen peroxide as a paracrine vascular mediator: regulation and signaling leading to dysfunction. *Experimental biology and medicine (Maywood, N.J* **231**, 237-251 (2006).

162.Cai, H., Griendling, K.K. & Harrison, D.G. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends in pharmacological sciences* **24**, 471-478 (2003).

163.Zafari, A.M. *et al.* Role of NADH/NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy. *Hypertension* **32**, 488-495 (1998).

164.Seshiah, P.N. *et al.* Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circulation research* **91**, 406-413 (2002).

165.Clempus, R.E. & Griendling, K.K. Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovascular research* **71**, 216-225 (2006).

166.Kappert, K. *et al.* Antioxidants relieve phosphatase inhibition and reduce PDGF signaling in cultured VSMCs and in restenosis. *Arterioscler Thromb Vasc Biol* **26**, 2644-2651 (2006).

167.Taniyama, Y. *et al.* Role of p38 MAPK and MAPKAPK-2 in angiotensin II-induced Akt activation in vascular smooth muscle cells. *Am J Physiol Cell Physiol* **287**, C494-499 (2004).

168.Lambeth, J.D. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* **4**, 181-189 (2004).

169.Sumimoto, H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. *The FEBS journal* **275**, 3249-3277 (2008).

170.Lassegue, B. & Clempus, R.E. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* **285**, R277-297 (2003).

171.Brandes, R.P. & Schroder, K. Composition and functions of vascular nicotinamide adenine dinucleotide phosphate oxidases. *Trends in cardiovascular medicine* **18**, 15-19 (2008).

172.Bedard, K. & Krause, K.H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews* **87**, 245-313 (2007).

173.Schulz, E. & Munzel, T. NOX5, a new "radical" player in human atherosclerosis? *Journal of the American College of Cardiology* **52**, 1810-1812 (2008).

174.Leto, T.L., Morand, S., Hurt, D. & Ueyama, T. Targeting and Regulation of Reactive Oxygen Species Generation by Nox Family NADPH Oxidases. *Antioxidants & redox signaling* (2009).

175.Brandes, R.P. & Kreuzer, J. Vascular NADPH oxidases: molecular mechanisms of activation. *Cardiovascular research* **65**, 16-27 (2005).

176.Griendling, K.K., Minieri, C.A., Ollerenshaw, J.D. & Alexander, R.W. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circulation research* **74**, 1141-1148 (1994).

177.Dikalov, S.I. *et al.* Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. *Free radical biology & medicine* **45**, 1340-1351 (2008).

178.Ushio-Fukai, M. Compartmentalization of Redox Signaling through NADPH Oxidase-derived ROS. *Antioxidants & redox signaling* (2008).

179.Garrido, A.M. & Griendling, K.K. NADPH oxidases and angiotensin II receptor signaling. *Molecular and cellular endocrinology* **302**, 148-158 (2009).

180.Guzik, T.J. & Griendling, K. NADPH oxidases - molecular understanding finally reaching the clinical level? *Antioxidants & redox signaling* (2009).

181.Ushio-Fukai, M. *et al.* cAbl tyrosine kinase mediates reactive oxygen species- and caveolin-dependent AT1 receptor signaling in vascular smooth muscle: role in vascular hypertrophy. *Circulation research* **97**, 829-836 (2005).

182.Menshikov, M. *et al.* Urokinase plasminogen activator stimulates vascular smooth muscle cell proliferation via redox-dependent pathways. *Arterioscler Thromb Vasc Biol* **26**, 801-807 (2006).

183.Suh, Y.A. *et al.* Cell transformation by the superoxide-generating oxidase Mox1. *Nature* **401**, 79-82 (1999).

184.Schroder, K. *et al.* Nox1 mediates basic fibroblast growth factor-induced migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **27**, 1736-1743 (2007).

185.Weber, D.S. *et al.* Phosphoinositide-dependent kinase 1 and p21-activated protein kinase mediate reactive oxygen species-dependent regulation of platelet-derived growth factor-induced smooth muscle cell migration. *Circulation research* **94**, 1219-1226 (2004).

186.Lassegue, B. *et al.* Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circulation research* **88**, 888-894 (2001).

187.Matsuno, K. *et al.* Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice. *Circulation* **112**, 2677-2685 (2005).

188.Gavazzi, G. *et al.* Decreased blood pressure in NOX1-deficient mice. *FEBS letters* **580**, 497-504 (2006).

189.Gorin, Y. *et al.* Nox4 mediates angiotensin II-induced activation of Akt/protein kinase B in mesangial cells. *Am J Physiol Renal Physiol* **285**, F219-229 (2003).

190.Clempus, R.E. *et al.* Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. *Arterioscler Thromb Vasc Biol* **27**, 42-48 (2007).

191.Hilenski, L.L., Clempus, R.E., Quinn, M.T., Lambeth, J.D. & Griendling, K.K. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **24**, 677-683 (2004).

192.Sanz, J. & Fayad, Z.A. Imaging of atherosclerotic cardiovascular disease. *Nature* **451**, 953-957 (2008).

193.Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **72**, 248-254 (1976).

194.Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685 (1970).

195.Jakubowski, W. & Bartosz, G. 2,7-dichlorofluorescin oxidation and reactive oxygen species: what does it measure? *Cell biology international* **24**, 757-760 (2000).

196.Zhou, M., Diwu, Z., Panchuk-Voloshina, N. & Haugland, R.P. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Analytical biochemistry* **253**, 162-168 (1997).

197.Persson, C. *et al.* Preferential oxidation of the second phosphatase domain of receptor-like PTP-alpha revealed by an antibody against oxidized protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* **101**, 1886-1891 (2004).

198.Groen, A. *et al.* Differential oxidation of protein-tyrosine phosphatases. *The Journal of biological chemistry* **280**, 10298-10304 (2005).

199.Tang, D.D. & Anfinogenova, Y. Physiologic properties and regulation of the actin cytoskeleton in vascular smooth muscle. *Journal of cardiovascular pharmacology and therapeutics* **13**, 130-140 (2008).

200.Rahman, I., Biswas, S.K. & Kirkham, P.A. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochemical pharmacology* **72**, 1439-1452 (2006).

201.Majander, A., Finel, M. & Wikstrom, M. Diphenyleneiodonium inhibits reduction of iron-sulfur clusters in the mitochondrial NADH-ubiquinone oxidoreductase (Complex I). *The Journal of biological chemistry* **269**, 21037-21042 (1994).

202.Li, Y., Lappas, G. & Anand-Srivastava, M.B. Role of oxidative stress in angiotensin II-induced enhanced expression of Gi(alpha) proteins and adenylyl cyclase signaling in A10 vascular smooth muscle cells. *American journal of physiology* **292**, H1922-1930 (2007).

203.DeYulia, G.J., Jr. & Carcamo, J.M. EGF receptor-ligand interaction generates extracellular hydrogen peroxide that inhibits EGFR-associated protein tyrosine phosphatases. *Biochem Biophys Res Commun* **334**, 38-42 (2005).

204.Lambeth, J.D., Krause, K.H. & Clark, R.A. NOX enzymes as novel targets for drug development. *Seminars in immunopathology* **30**, 339-363 (2008).

205.Zuo, L., Ushio-Fukai, M., Hilenski, L.L. & Alexander, R.W. Microtubules regulate angiotensin II type 1 receptor and Rac1 localization in caveolae/lipid rafts: role in redox signaling. *Arterioscler Thromb Vasc Biol* **24**, 1223-1228 (2004).

206.Azios, N.G. *et al.* Estrogen and resveratrol regulate Rac and Cdc42 signaling to the actin cytoskeleton of metastatic breast cancer cells. *Neoplasia (New York, N.Y* **9**, 147-158 (2007).

207.Rey, F.E., Cifuentes, M.E., Kiarash, A., Quinn, M.T. & Pagano, P.J. Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O(2)(-) and systolic blood pressure in mice. *Circulation research* **89**, 408-414 (2001).

208.Diatchuk, V., Lotan, O., Koshkin, V., Wikstroem, P. & Pick, E. Inhibition of NADPH oxidase activation by 4-(2-aminoethyl)-benzenesulfonyl fluoride and related compounds. *The Journal of biological chemistry* **272**, 13292-13301 (1997).

209.Sato, M. *et al.* c-Src and hydrogen peroxide mediate transforming growth factor-beta1-induced smooth muscle cell-gene expression in 10T1/2 cells. *Arterioscler Thromb Vasc Biol* **25**, 341-347 (2005).

210.Tsoupras, A.B. *et al.* Characterization of the de novo biosynthetic enzyme of platelet activating factor, DDT-insensitive cholinephosphotransferase, of human mesangial cells. *Mediators of inflammation* **2007**, 27683 (2007).

211.Wildroudt, M.L. & Freeman, E.J. Regulation of Akt by arachidonic acid and phosphoinositide 3-kinase in angiotensin II-stimulated vascular smooth muscle cells. *Biochimica et biophysica acta* **1761**, 11-16 (2006).

212.Bolli, R. *et al.* Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. *Circulation research* **65**, 607-622 (1989).

213.Chakraborti, S., Batabyal, S.K. & Chakraborti, T. Role of hydroxyl radical in the stimulation of arachidonic acid release caused by H2O2 in pulmonary smooth muscle cells: protective effect of anion channel blocker. *Molecular and cellular biochemistry* **146**, 91-98 (1995).

214.Chen, X.L., Zhang, Q., Zhao, R. & Medford, R.M. Superoxide, H2O2, and iron are required for TNF-alpha-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase. *American journal of physiology* **286**, H1001-1007 (2004).

215.Baker, K. *et al.* Synthetic combined superoxide dismutase/catalase mimetics are protective as a delayed treatment in a rat stroke model: a key role for reactive oxygen species in ischemic brain injury. *The Journal of pharmacology and experimental therapeutics* **284**, 215-221 (1998).

216.Xiao, L. *et al.* Role of reactive oxygen species and NAD(P)H oxidase in alpha(1)-adrenoceptor signaling in adult rat cardiac myocytes. *Am J Physiol Cell Physiol* **282**, C926-934 (2002).

217.Ross, S.H. *et al.* Differential redox regulation within the PTP superfamily. *Cellular signalling* **19**, 1521-1530 (2007).

218.Denu, J.M. & Tanner, K.G. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* **37**, 5633-5642 (1998).

219.Persson, C., Kappert, K., Engstrom, U., Ostman, A. & Sjoblom, T. An antibody-based method for monitoring in vivo oxidation of protein tyrosine phosphatases. *Methods* **35**, 37-43 (2005).

220.Yart, A. *et al.* A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of ras and mitogen-activated protein kinases by epidermal growth factor. *The Journal of biological chemistry* **276**, 8856-8864 (2001).

221.Frojdo, S., Cozzone, D., Vidal, H. & Pirola, L. Resveratrol is a class IA phosphoinositide 3-kinase inhibitor. *The Biochemical journal* **406**, 511-518 (2007).

222.Grozinger, C.M., Chao, E.D., Blackwell, H.E., Moazed, D. & Schreiber, S.L. Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. *The Journal of biological chemistry* **276**, 38837-38843 (2001).

223.Oak, M.H., Bedoui, J.E., Madeira, S.V., Chalupsky, K. & Schini-Kerth, V.B. Delphinidin and cyanidin inhibit PDGF(AB)-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK. *British journal of pharmacology* **149**, 283-290 (2006).

224.Liu, Y. & Liu, G. Isorhapontigenin and resveratrol suppress oxLDL-induced proliferation and activation of ERK1/2 mitogen-activated protein kinases of bovine aortic smooth muscle cells. *Biochemical pharmacology* **67**, 777-785 (2004).

225.Opie, L.H. & Lecour, S. The red wine hypothesis: from concepts to protective signalling molecules. *European heart journal* **28**, 1683-1693 (2007).

226.Gamou, S. & Shimizu, N. Hydrogen peroxide preferentially enhances the tyrosine phosphorylation of epidermal growth factor receptor. *FEBS letters* **357**, 161-164 (1995).

227.Bae, Y.S. *et al.* Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *The Journal of biological chemistry* **272**, 217-221 (1997).

228.Hanasaki, Y., Ogawa, S. & Fukui, S. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free radical biology & medicine* **16**, 845-850 (1994).

229.Ushio-Fukai, M. *et al.* Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **21**, 489-495 (2001).

230.Hingtgen, S.D. *et al.* Nox2-containing NADPH oxidase and Akt activation play a key role in angiotensin II-induced cardiomyocyte hypertrophy. *Physiological genomics* **26**, 180-191 (2006).

231.Park, D.W. *et al.* Resveratrol inhibits foam cell formation via NADPH oxidase 1- mediated reactive oxygen species and monocyte chemotactic protein-1. *Experimental & molecular medicine* **41**, 171-179 (2009).

232.Chen, K., Kirber, M.T., Xiao, H., Yang, Y. & Keaney, J.F., Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *The Journal of cell biology* **181**, 1129-1139 (2008).

233.Nauseef, W.M. Biological roles for the NOX family NADPH oxidases. *The Journal of biological chemistry* **283**, 16961-16965 (2008).

234.Choi, H. *et al.* Mechanism of angiotensin II-induced superoxide production in cells reconstituted with angiotensin type 1 receptor and the components of NADPH oxidase. *The Journal of biological chemistry* **283**, 255-267 (2008).

235.Wang, L., Lopaschuk, G.D. & Clanachan, A.S. H(2)O(2)-induced left ventricular dysfunction in isolated working rat hearts is independent of calcium accumulation. *Journal of molecular and cellular cardiology* **45**, 787-795 (2008).

236.Baumer, A.T. *et al.* Phosphatidylinositol 3-kinase-dependent membrane recruitment of Rac-1 and p47phox is critical for alpha-platelet-derived growth factor receptor-induced production of reactive oxygen species. *The Journal of biological chemistry* **283**, 7864-7876 (2008).

237.Ushio-Fukai, M. *et al.* Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *The Journal of biological chemistry* **274**, 22699-22704 (1999).

238.Aldieri, E. *et al.* Classical inhibitors of NOX NAD(P)H oxidases are not specific. *Current drug metabolism* **9**, 686-696 (2008).

239.Peiro, C. *et al.* Glycosylated human oxyhaemoglobin activates nuclear factor-kappaB and activator protein-1 in cultured human aortic smooth muscle. *British journal of pharmacology* **140**, 681-690 (2003).

240.Hansen-Hagge, T.E. *et al.* Transmission of oxLDL-derived lipid peroxide radicals into membranes of vascular cells is the main inducer of oxLDL-mediated oxidative stress. *Atherosclerosis* **197**, 602-611 (2008).

241.Lacerda, L., Smith, R.M., Opie, L. & Lecour, S. TNFalpha-induced cytoprotection requires the production of free radicals within mitochondria in C2C12 myotubes. *Life sciences* **79**, 2194-2201 (2006).

242.Mountain, D.J., Singh, M., Menon, B. & Singh, K. Interleukin-1beta increases expression and activity of matrix metalloproteinase-2 in cardiac microvascular endothelial cells: role of PKCalpha/beta1 and MAPKs. *Am J Physiol Cell Physiol* **292**, C867-875 (2007).

243.Wen, X. & Walle, T. Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug metabolism and disposition: the biological fate of chemicals* **34**, 1786-1792 (2006).

244.Wen, X. & Walle, T. Methylation protects dietary flavonoids from rapid hepatic metabolism. *Xenobiotica; the fate of foreign compounds in biological systems* **36**, 387-397 (2006).

245.Pervaiz, S. & Holme, A.L. Resveratrol: Its Biological Targets and Functional Activity. *Antioxidants & redox signaling* (2009).

246.Chen, C.H. *et al.* Reactive oxygen species generation is involved in epidermal growth factor receptor transactivation through the transient oxidization of Src homology 2-containing tyrosine phosphatase in endothelin-1 signaling pathway in rat cardiac fibroblasts. *Mol Pharmacol* **69**, 1347-1355 (2006).

247.Godeny, M.D. *et al.* The N-terminal SH2 domain of the tyrosine phosphatase, SHP-2, is essential for Jak2-dependent signaling via the angiotensin II type AT1 receptor. *Cellular signalling* **19**, 600-609 (2007).

248.Saxton, T.M. *et al.* Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2. *The EMBO journal* **16**, 2352-2364 (1997).

249.Doan, T., Farmer, P., Cooney, T. & Ali, M.S. Selective down-regulation of angiotensin II receptor type 1A signaling by protein tyrosine phosphatase SHP-2 in vascular smooth muscle cells. *Cellular signalling* **16**, 301-311 (2004).

250.Zito, C.I., Qin, H., Blenis, J. & Bennett, A.M. SHP-2 regulates cell growth by controlling the mTOR/S6 kinase 1 pathway. *The Journal of biological chemistry* **282**, 6946-6953 (2007).

251.Ivins Zito, C., Kontaridis, M.I., Fornaro, M., Feng, G.S. & Bennett, A.M. SHP-2 regulates the phosphatidylinositide 3'-kinase/Akt pathway and suppresses caspase 3-mediated apoptosis. *Journal of cellular physiology* **199**, 227-236 (2004).

252.Wu, C.J. *et al.* The tyrosine phosphatase SHP-2 is required for mediating phosphatidylinositol 3-kinase/Akt activation by growth factors. *Oncogene* **20**, 6018-6025 (2001).

253.Sampaio, C. *et al.* Signal strength dictates phosphoinositide 3-kinase contribution to Ras/extracellular signal-regulated kinase 1 and 2 activation via differential Gab1/Shp2 recruitment: consequences for resistance to epidermal growth factor receptor inhibition. *Molecular and cellular biology* **28**, 587-600 (2008).

254.Shah, B.H. *et al.* Differential pathways of angiotensin II-induced extracellularly regulated kinase 1/2 phosphorylation in specific cell types: role of heparin-binding epidermal growth factor. *Molecular endocrinology (Baltimore, Md* **18**, 2035-2048 (2004).

255.Andresen, B.T., Linnoila, J.J., Jackson, E.K. & Romero, G.G. Role of EGFR transactivation in angiotensin II signaling to extracellular regulated kinase in preglomerular smooth muscle cells. *Hypertension* **41**, 781-786 (2003).

256.Yogi, A. *et al.* Endothelin-1, but not Ang II, activates MAP kinases through c-Src independent Ras-Raf dependent pathways in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **27**, 1960-1967 (2007).

257.Bokemeyer, D., Schmitz, U. & Kramer, H.J. Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor. *Kidney international* **58**, 549-558 (2000).

258.Venkatesan, B. *et al.* Resveratrol inhibits PDGF receptor mitogenic signaling in mesangial cells: role of PTP1B. *Faseb J* **22**, 3469-3482 (2008).

259.Lin, H.Y. *et al.* Integrin alphaVbeta3 contains a receptor site for resveratrol. *Faseb J* **20**, 1742-1744 (2006).

G APPENDIX

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
ddH_2O	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

ERK	Extracellular signal-regulated kinase
F	
FACS	Fluorescence-activated cell sorter
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box-O transcription factors
G	
Gab	Grb-2associated binder (Gab)
GAP	GTPase activating protein
Grb	Growth factor receptor binding protein
GSK	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
H	
HBSS	Hank's balanced salt solution
H_2DCF	2′,7′-dichlorodihydrofluorescein
H_2DCF-DA	2′,7′-dichlorodihydrofluorescein-diacetate
HDAC	Histone deacetylase
HEPES	N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid)
I	
IAA	Iodoacetic acid
IP	Immunoprecipitation
J	
JNK	c-Jun N-terminal kinase
K	
kDA	kilo Dalton
L	
LDL	Low density lipoprotein
LMW-PTP	Low molecular weight protein-tyrosine phosphatase
M	
MAPK	Mitogen activated protein kinase
Mc	Monoclonal
MnIIITM	Mn(III)terakis(1- methyl-4-pyridyl) porphyrin pentachloride

MPG	N-(2-mercaptopropionyl)-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

S

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodim dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SH2 domain	Src-homology 2 domain
SHIP	SH2-containing inositol 5-phosphatase
Shp2	SH2 domain-containing phosphatase 2
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
SOS	Son of sevenless

T

TBS-T	Tris-buffered saline containing Tween 20
T/E	Trypsin/EDTA solution
TEMED	N,N,N′,N′-tetramethylethylene diamine+
Thr	Threonine
Tyr	Tyrosin

U

U	Units

V

VSMC	Vascular smooth muscle cells

W

WM	Wortmannin

2. Publications

Cornelia E. Schreiner, Mario Kumerz, Julia Gesslbauer, Thomas Erker, Atanas G. Atanasov, Elke H. Heiss, Verena M. Dirsch. Resveratol-mediated inhibition of Akt phosphorylation in Ang II or EGF-activated VSMC: role of ROS and NADPH oxidases 1 and 4.
Manuscript in preparation

3. Poster Presentations

Cornelia E. Schreiner, Mario Kumerz, Julia Gesslbauer, Atanas G. Atanasov, Elke H. Heiss, Thomas Erker, Verena M. Dirsch. Resveratrol-mediated inhibition of Ang II-induced Akt phosphorylation in VSMC – is it an antioxidant activity? 21st Scientific Congress of the ÖPhG, Vienna (Austria), 16 - 18 April 2009

Cornelia E. Schreiner, Mario Kumerz, Atanas G. Atanasov, Elke H. Heiss, Verena M. Dirsch. Inhibition of growth factor mediated Akt phosphorylation in vascular smooth muscle cells by resveratrol - the contribution of SH2 domain containing phosphatase 2 and reactive oxygen species. 14th Scientific Symposium of the Austrian Pharmacological Society (APHAR), Innsbruck (Austria), 20 - 22 November 2008

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, Praque (Czech Republic), 4 - 9 July 2009 (*invited lecuture*)

5. Curriculum Vitae

Personal information

Present address	Dettergasse 1/2/4 A-1160 Vienna Austria
Date and place of birth	September 12th, 1979 Amstetten, Austria
Nationality	Austria

Education

11/2005 – present	PhD-Thesis at the Department of Pharmacognosy, University of Vienna "*Mechanistic studies on resveratrol and its influence on angiotensin II- and epidermal growth factor-induced signaling pathways in vascular smooth muscle cells"*
07/2005	Graduation (academic degree: *Magister rerum naturalium*)
01/2004 – 02/2005	Diploma-Thesis "*Effects Of Growth Factor Suppression By Gene Silencing On Growth Of Human Colon Cancer Xenografts In Mice"* at the Medical University of Vienna
10/1999 – 07/2005	Study of genetics and microbiology at the University of Vienna
10/1998 - 10/1999	Study of Nutrition Sciences at the University of Vienna
09/1993 – 06/1998	Higher Secondary School for Culture and Congress Management

International stays

09/2006	Scientific stay in the group of Prof. Östman, Karolinska Institute, Stockholm
09/2002 – 02/2003	Exchange student at the University of Manchester

Teaching experience

SS 2005 - SS 2009	Participating in the lab course "Methods for identification and characterisation of drug providing organisms"
WS 2006/07 - WS 2008/09	Participating in the lab course "Microbiology and Hygiene
WS 2008/2009	Supervision of a diploma student

Other activities

07/2007 – 07/2009	Representative of the Doctoral students of Natural Sciences

6. Acknowledgements / Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die zur Entstehung dieser Arbeit beigetragen haben.

Zuerst möchte ich mich bei meiner Chefin und Betreuerin Prof. Verena Dirsch bedanken, dass Sie mir die Chance gegeben hat, meine Doktorarbeit in Ihrer Gruppe durchzuführen. Zusätzlich hat Sie mir sozusagen Ihr „erstgeborenes“ Projekt überlassen und mich immer mit vielen Ideen und Enthusiasmus bei dieser Arbeit unterstützt.

Prof. Erker und seinen Mitarbeitern möchte ich für die Synthese der Resveratrol - Derivate ganz herzlich danken.

Ein weiterer Dank gebührt Elke, die mir die ganzen Jahre hindurch mit praktischen Tipps und Ratschlägen aller Art die Arbeit sehr erleichtert hat und mir in jeglicher Hinsicht geholfen hat.
Auch Ata möchte ich für seinen Ideen und Gedanken zu diesem Projekt danken, sie haben einen wichtigen Teil hierzu beigetragen.

Auch möchte ich meinen Vorgängern, mit denen ich auch die ersten Jahre gemeinsam im Labor verbringen durfte - Andrea, Christoph und Yvonne - da sie maßgeblich am Aufbau des Labors beteiligt waren. Nur dank Euch konnte ich gleich in meinen ersten Wochen mit Western Blots beginnen. Andrea, Danke für die Einführung in die Arbeit mit VSMC. Christoph, ich erinnere mich gerne an unsere gemeinsame Zeit im Büro und Yvonne - Danke für Deine Unterstützung. Vor allem aber habt Ihr mir vorgeführt, dass eine Doktorarbeit auch ein Ende hat.

Mario, meinem „Mitkämpfer“ an diesem Projekt, vielen Dank für Deine Beiträge zu dieser Arbeit - die fachlichen Gespräche und den experimentellen Input. Irene, danke für alle fachlichen Diskussionen.

Meinem jetzigen Zimmerkollegen Helge, danke für Hilfestellungen aller Art und für die vielen Tees, die ich dank Dir kennen lernen konnte.

Tenzi, Daniel und Rebecca, vielen Dank für Eure immerwährende Unterstützung im Labor. Der Laboralltag hat nur deshalb so reibungslos funktioniert, weil Ihr immer ein Auge auf den laufenden Betrieb hattet und auch mit Eurer netten Art viel zum angenehmen Klima beigetragen habt.

Ich möchte auch ganz herzlich meiner Diplomatin Julia danken, die ebenfalls experimentell zu dieser Arbeit beigetragen hat.

Natürlich möchte ich mich auch bei allen anderen Kollegen des Departments bedanken, die durch ihre Freundlichkeit die Arbeit hier sehr angenehm gemacht haben.

Liebe Mama, lieber Papa, Euch gebührt ein großes Dankeschön. Vielen Dank für die bedingungslose und liebevolle Unterstützung während der letzten Jahre. Ihr habt immer daran geglaubt, dass diese Arbeit einmal beendet sein wird. Dieser unerschütterlichen Glauben in mich hat mich durchhalten lassen.

Zu Guter letzt möchte ich meinem Freund Daniel von ganzen Herzen danken. Du bist mir die ganzen Jahre über beigestanden und hast durch Unterstützung jeglicher Art sehr viel zu dieser Arbeit beigetragen. Vielen Dank für alles!