

Research Article

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Resveratrol modulates signalling to inhibit vascular smooth muscle cell proliferation induced by angiotensin II and high glucose

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Abstract

Objectives: The proliferation of vascular smooth muscle cells (VSMCs) induced by hyperglycemia plays a pivotal role in the development of atherosclerosis and restenosis. This study aims to examine the impact of angiotensin II (Ang II) and high glucose on VSMC proliferation and the phosphorylation status of key signalling proteins, specifically ERK1/2, Akt, and STAT3. Furthermore, we assess the inhibitory effects of resveratrol, a polyphenolic compound, on these signalling pathways.

Methods: Primary vascular smooth muscle cells (VSMCs) isolated from rat aortas were cultured in both standard media (SM: 5.5 mM) and high glucose media (HGM: 25 mM) and then treated with Ang II (100 nM). Proliferation was assessed using the WST-1 assay, and protein analysis was performed through immunoblotting.

Results: Ang II increased VSMC proliferation by 39 % in standard glucose environments and 17 % in high glucose environments. Resveratrol effectively suppressed Ang II-induced VSMC proliferation in both media. Furthermore, resveratrol inhibited the phosphorylation of ERK1/2 and Akt. Ang II also induced STAT3 phosphorylation by 29 and 18.5 % in SM and HGM, respectively. However, resveratrol treatment reduced STAT3 phosphorylation to control levels.

Conclusions: These findings demonstrated that resveratrol reduces VSMC proliferation induced by Ang II and high glucose conditions, exerting its inhibitory effects by

suppressing ERK1/2, Akt, and STAT3 phosphorylation. These results provide valuable insights into the cardioprotective properties of resveratrol.

Keywords: vascular smooth muscle cell; resveratrol; proliferation; cardiovascular; angiotensin II

Introduction

Diabetes mellitus affects a significant number of individuals, with its primary complication being cardiovascular disease (CVD). Individuals with diabetes often encounter accelerated atherosclerosis [1]. Hyperglycemia, a key contributor to CVD development among people with diabetes, induces vascular smooth muscle cells (VSMCs) proliferation by instigating structural and functional abnormalities in the vascular wall. Normally, VSMCs display contractile characteristics and maintain low proliferation rates. However, in response to external stimuli such as Ang II or hyperglycemia, they can transform phenotypically into a secretory and highly proliferative state, contributing to CVD pathologies like atherosclerosis and restenosis [2]. Hyperglycemia achieves this effect by promoting the production of reactive oxygen species (ROS), enhancing the mitogenic response mediated by intracellular signalling pathways, including mitogen-activated protein kinases (MAPKs) and Akt, and thereby facilitating the proliferation of VSMCs [3].

Our understanding of Ang II has evolved, recognising it not only as a crucial player in the renin-angiotensin system (RAS) but also as a vasoactive agent with growth factor-like effects capable of triggering numerous intracellular signalling pathways. Ang II's binding to the type I receptor (AT1R), known to mediate the pathophysiological effects of Ang II, activates various intracellular signalling pathways, including ERK1/2, Akt, the JAK-STAT pathway, NADPH oxidase-mediated ROS production, and epidermal growth factor receptor (EGFR) activation. These activations contribute to processes implicated in CVD, such as endothelial dysfunction, vascular inflammation, and fibrosis, by affecting cellular mechanisms, including ROS production

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and VSMC proliferation [4]. Increased VSMC proliferation is observed in early atherogenesis, vascular injury, and ageing rodents compared to their young counterparts [5]. The role of the RAS in the pathogenesis of chronic vascular diseases and the significance of Ang II as a major factor influencing VSMC proliferation are well recognised. Therefore, targeting the prevention of VSMC proliferation is crucial to mitigating associated CVD pathologies.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a polyphenolic molecule abundant in red grapes, is renowned for its antioxidant and cardioprotective properties. Studies have demonstrated that resveratrol reduces hyperglycemia and insulin resistance in animal models, lowers blood pressure in hypertension, diminishes oxidative damage and inflammation, and exerts beneficial effects against atherosclerosis [6, 7]. While the primary molecular targets of resveratrol are sirtuins and AMPK activation, it has also been associated with several signalling pathways, including cyclooxygenases, NF- κ B, phosphodiesterases, MAP kinases, and PI3K/Akt [8]. Resveratrol has been shown to downregulate AT1R expression in VSMCs [9]. Conversely, Kim and colleagues have reported the absence of this effect [10]. Moreover, the effects of resveratrol on Akt and ERK1/2 phosphorylation remain contentious, with some studies indicating the inhibition of Akt phosphorylation [11–13], others reporting no effect [14, 15], and even some demonstrating the activation of Akt phosphorylation [16, 17] and the inhibition of apoptosis [18]. Similar discrepancies exist regarding ERK1/2 phosphorylation [11, 14, 17]. Furthermore, although VSMC proliferation in atherogenesis shares similarities with the processes observed in cancer development, the impact of Ang II stimulation on STAT3 phosphorylation and the influence of resveratrol on this phosphorylation under normal and hyperglycemic conditions in VSMCs have not been extensively investigated.

Therefore, the present study aimed to examine the effects of Ang II on proliferation, ERK1/2, Akt, and STAT3 phosphorylation in VSMCs under standard (SM, 5.5 mM) and high glucose (HGM, 25 mM) media. Additionally, we investigated the effects of the AT1R antagonist losartan, the EGFR inhibitor AG1478, the MAPK inhibitor PD98059, and resveratrol on these phosphoproteins and VSMC proliferation.

Materials and methods

Ethics statement

This study adhered to the ethical guidelines outlined in the Declaration of Helsinki. Consent was granted by the Ethics Committee of Akdeniz University (Approval Code: 38, Date: May 23, 2016).

Reagents

DMEM, HEPES, elastase, collagenase, soy trypsin inhibitor, foetal bovine serum, L-glutamine, penicillin-streptomycin, resveratrol, and angiotensin II were procured from Sigma (St. Louis, MO, USA). Trypsin-EDTA was obtained from Biochrom AG (Berlin, Germany), and the inhibitors were sourced from Calbiochem (San Diego, CA, USA). The AT1R antagonist losartan was acquired from Merck (Darmstadt, Germany). The protein measurement kit and ECL reagent were ordered from Bio-Rad (Hercules, CA, USA). The primary antibody for p-ERK1/2 was obtained from Sigma, while the primary antibodies for p-STAT3 and p-Akt, as well as the secondary antibodies (anti-mouse Ig and anti-rabbit Ig), were purchased from Cell Signaling (Beverly, MA, USA).

Isolation and primary culture of rat VSMCs

This study received approval from the Akdeniz University Local Committee on Animal Research Ethics. Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of male Wistar rats (250–350 g) following previously described procedures [19]. VSMCs were cultivated in DMEM supplemented with 10 % (v/v) foetal calf serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. The VSMCs used in this study were in passages 3–5. A day before each experiment, cultures with 80 % confluence were incubated with serum-free media. Cells in SM and HGM were maintained in their respective serum-free conditions.

Quantification of cellular proliferation

Cellular proliferation was determined through spectrophotometry using a soluble WST-1 tetrazolium salt (4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzenedisulfonate) obtained from Roche (Mannheim, Germany). Cells were harvested through trypsin-EDTA treatment, followed by centrifugation. The resulting pellet was re-suspended in the medium, and approximately 4000 cells per well were seeded in a 96-well plate. After introducing the inhibitors into the medium, a 30-min incubation ensued. Subsequently, Ang II was added, and further incubation took place. At the end of the incubation period, the WST-1 reagent was added to each well and incubated at 37 °C for 4 h. Following colour development, absorbance values were measured at a wavelength of 440 nm using a spectrophotometer (μ Quant, Biotek, Agilent). Each well underwent measurements at least eight times ($n=8$).

Immunoblot analysis

Immunoblotting procedures were conducted as previously outlined [19]. In brief, VSMCs at 80 % confluence were cultured overnight in serum-free DMEM at 37 °C. Subsequently, cells were subjected to a 30-min pretreatment with inhibitors before stimulation. After the incubation period, cellular lysis was performed, and protein concentration was determined (Thermo Fisher Scientific, Waltham, MA, USA). Samples were standardised for protein content, and 50 μ g of protein was loaded onto SDS-PAGE gels. For p-ERK1/2, 12 % gels were used, while 10 % gels were employed for p-Akt. The gel content was then transferred to a membrane, followed by an overnight incubation of the membrane with primary antibodies (1:1,000–2,000) at 4 °C. Subsequent probing of the blots was carried out with an HRP-conjugated secondary antibody

(1:5,000). Immunoreactive protein detection was achieved using an ECL reagent (Thermo Fisher Scientific, Waltham, MA, USA). The AT1R antagonist losartan was administered at a concentration of 10 μ M, the EGFR kinase inhibitor AG1478 at 1 μ M, the ERK1/2 inhibitor PD98059 at 1 μ M, and resveratrol at concentrations of 50 and 100 μ M. These inhibitors were introduced to the medium for at least 30 min before adding the stimulant.

Data analysis

The data is presented as the mean \pm SEM, with a significance level of $p\leq 0.05$. Multiple t-tests were employed (one for each row) to assess the significance between various groups using GraphPad Prism (version 8.0.1). Band density in the Western blot images was quantified using ImageJ software (version 1.52a).

Results

Angiotensin II enhances proliferation more efficiently in high glucose media

Cells cultured in standard media (SM) and high glucose media (HGM) were exposed to Ang II treatment at a concentration of 100 nM for 24 h to assess its impact on proliferation. In the HGM group, the cells were exposed to a high glucose environment for 48 h before receiving Ang II treatment. The proliferation levels in vascular smooth muscle cells (VSMCs) were significantly increased after 24 h of incubation with Ang II. Specifically, there was a 39 % increase in SM; in HGM, the increase was 17 % ($p\leq 0.01$, as shown in Figure 1). Cells in the HGM group exhibited nearly 1.45-fold increased proliferation compared to the SM group ($p\leq 0.01$, Figure 1A). The AT1R antagonist losartan and the EGFR inhibitor AG1478 effectively suppressed the increased proliferation to control levels in both environments ($p\leq 0.01$, Figure 1A). These results indicate that AT1R mediates Ang II-induced VSMC proliferation and that EGFR transactivation also contributes to proliferation.

VSMCs were pre-incubated with resveratrol for 24 h in both SM and HGM, followed by stimulation with Ang II for 24 and 48 h. In the 24-h group, resveratrol effectively inhibited the enhanced proliferation induced by Ang II in both experimental groups ($p\leq 0.05$ for 50 μ M, $p\leq 0.01$ for 100 μ M, as illustrated in Figure 1B). Similarly, in the 48-h group, resveratrol significantly attenuated the heightened proliferation in both conditions ($p\leq 0.05$ and $p\leq 0.01$, as depicted in Figure 1C). Notably, the inhibitory effect of 100 μ M resveratrol on Ang II-induced proliferation was more pronounced than that of 50 μ M resveratrol at both 24 and 48 h (Figure 1B and C).

Resveratrol mitigates ERK1/2 and Akt phosphorylation

The impact of Ang II on the phosphorylation of ERK1/2 and Akt in VSMCs was investigated with cells treated with Ang II for 5 min. Cells were divided into SM and HGM groups, with cells in the HGM group pre-incubated with high glucose for 48 h before Ang II administration. Ang II (5 min, 100 nM) increased ERK1/2 and Akt phosphorylation in both the SM and HGM groups ($p\leq 0.05$, Figure 2). The phosphorylation of ERK1/2 increased by an average of 1.6-fold, and Akt phosphorylation increased by 1.4-fold. Losartan and AG1478 effectively suppressed the increased phosphorylation of ERK1/2 and Akt induced by Ang II in both environments ($p\leq 0.05$ and 0.01, as shown in Figure 2A and B). Pre-treatment with 50 μ M resveratrol and PD98059 before Ang II stimulation reduced ERK1/2 and Akt phosphorylation below the control level ($p\leq 0.01$, Figure 2C and D).

Resveratrol suppresses Ang II-induced STAT3 phosphorylation

After we observed increased proliferation and stimulated ERK1/2 and Akt phosphorylation in VSMCs treated with Ang II and high glucose, we examined the effect of Ang II on STAT3 phosphorylation in SM and HGM. Ang II (100 nM, 5 min) triggered a 29 % increase in STAT3 phosphorylation in SM and an 18.5 % increase in HGM ($p\leq 0.01$, as shown in Figure 3). Losartan and AG1478 significantly inhibited the elevated STAT3 phosphorylation ($p\leq 0.05$) in the presence of Ang II (Figure 3A and B). Cells that received a 50 μ M resveratrol pre-treatment for 30 min before Ang II stimulation displayed a notable reduction in STAT3 phosphorylation in both the SM and HGM environments ($p\leq 0.05$, Figure 3C–E).

Discussion

Diabetes mellitus is associated with an elevated cardiovascular disease (CVD) risk, primarily due to accelerated atherosclerosis. Elevated blood glucose levels, a prominent contributor to CVD development in individuals with diabetes, stimulate the growth of vascular smooth muscle cells (VSMCs) and play a role in atherosclerosis and restenosis progression [20]. This study delves into the impact of Ang II and high glucose on VSMC proliferation, focusing on the phosphorylation of crucial signalling proteins involved

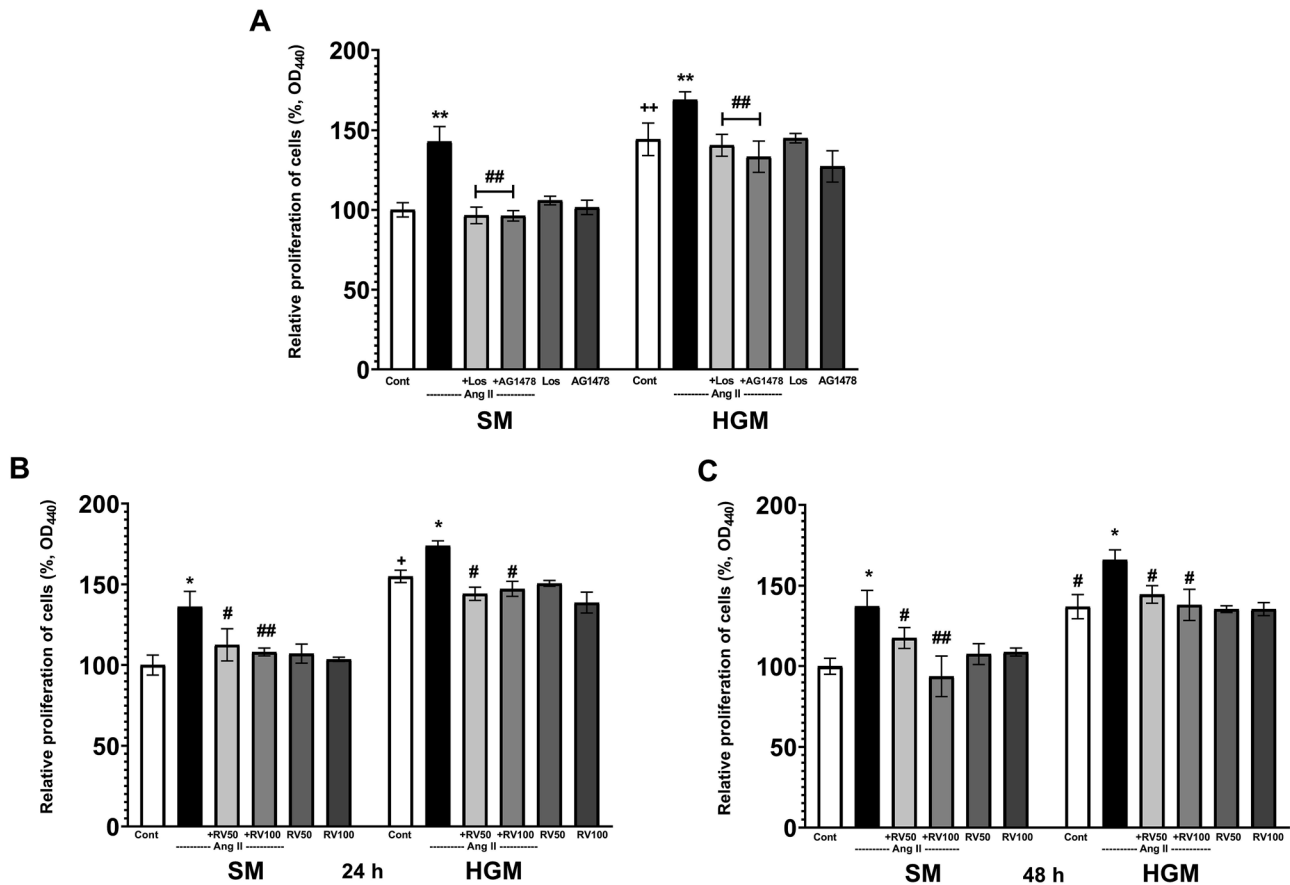


Figure 1: The effect of Ang II on the proliferation of VSMCs. Relative proliferation rates of the VSMCs. The cells were treated with agents for 30 min (losartan, AG1478, and resveratrol) before Ang II stimulation (100 nM, 24 h). The cells were either incubated in SM or HGM. (A) The proliferation rate of VSMCs and effects of losartan and AG1478 at 24 h in both media. (B) Resveratrol's effect on proliferation at 24 h. (C) Resveratrol's effect on proliferation at 48 h. Data are expressed as means \pm SEM, $n=8$. **: Significant compared to the control group with $p\leq 0.01$, ++: significant compared to the SM control group with $p\leq 0.01$, and # and ##: significant against Ang II with $p\leq 0.05$ and $p\leq 0.01$, respectively. Los, losartan; PD, PD98059; RV, resveratrol; SM, standard media; HGM, high glucose media.

in cell proliferation, namely ERK1/2, Akt, and STAT3. Additionally, we explore the potential inhibitory effects of resveratrol, a polyphenolic compound renowned for its antioxidant and cardioprotective properties, on these signalling pathways.

Ang II activates various intracellular signalling pathways via binding to the type I receptor (AT1R), encompassing MAP kinases, Akt, the JAK-STAT pathway, ROS production, and EGFR activation. These pathways contribute to CVD development by promoting endothelial dysfunction, vascular inflammation, and VSMC proliferation [4]. In alignment with prior research [21, 22], our results indicate a substantial increase in VSMC proliferation induced by Ang II in both standard and high glucose environments. This effect is mediated by AT1R activation, as the AT1R antagonist losartan effectively suppresses Ang II-induced proliferation. The restricted proliferation increase under high glucose conditions can be attributed to

the elevated baseline proliferation (45 %) in a high glucose environment, suggesting a possible threshold effect for Ang II-induced proliferation.

Additionally, the EGFR inhibitor AG1478 impedes VSMC proliferation, highlighting the involvement of EGFR trans-activation in this process. Resveratrol, extensively studied for its beneficial effects on various diseases, including diabetes, hypertension, and atherosclerosis [23], successfully curtails Ang II-induced VSMC proliferation in both standard and high glucose conditions. These findings align with previous studies reporting resveratrol's inhibitory effects on VSMC proliferation [24, 25]. Nevertheless, it is worth noting that Li et al. did not observe the effects of resveratrol on high glucose-exposed VSMCs, utilising concentrations of 10 and 30 μ M, with only 10 μ M affecting cell viability in standard conditions [26]. Guo and colleagues also did not detect an effect with 50 and 100 μ M resveratrol after 24 h in high glucose conditions [11].

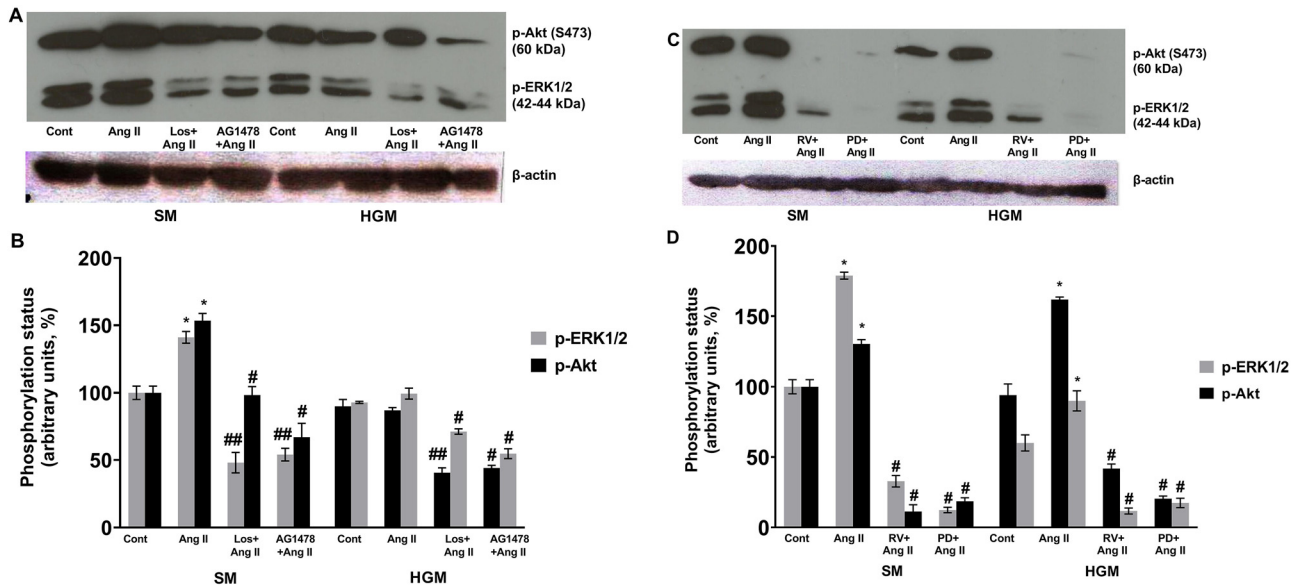


Figure 2: Ang II-induced phosphorylation status of ERK1/2 and Akt proteins. VSMCs were treated with inhibitors for 30 min, followed by stimulation with Ang II (100 nM, 5 min). (A, B) The effect of losartan and AG1478 on p-ERK1/2 and p-Akt upon induction by Ang II. (C, D) Resveratrol and PD98059's effect on p-ERK1/2 and p-Akt. Data are expressed as means \pm SEM, $n=3$. *: Significant compared to the control group with $p \leq 0.05$; +: significant compared to the SM control group with $p \leq 0.05$; and # and ##: significant against Ang II with $p \leq 0.05$ and $p \leq 0.01$, respectively. Los, losartan; PD, PD98059; RV, resveratrol; SM, standard media; HGM, high glucose media.

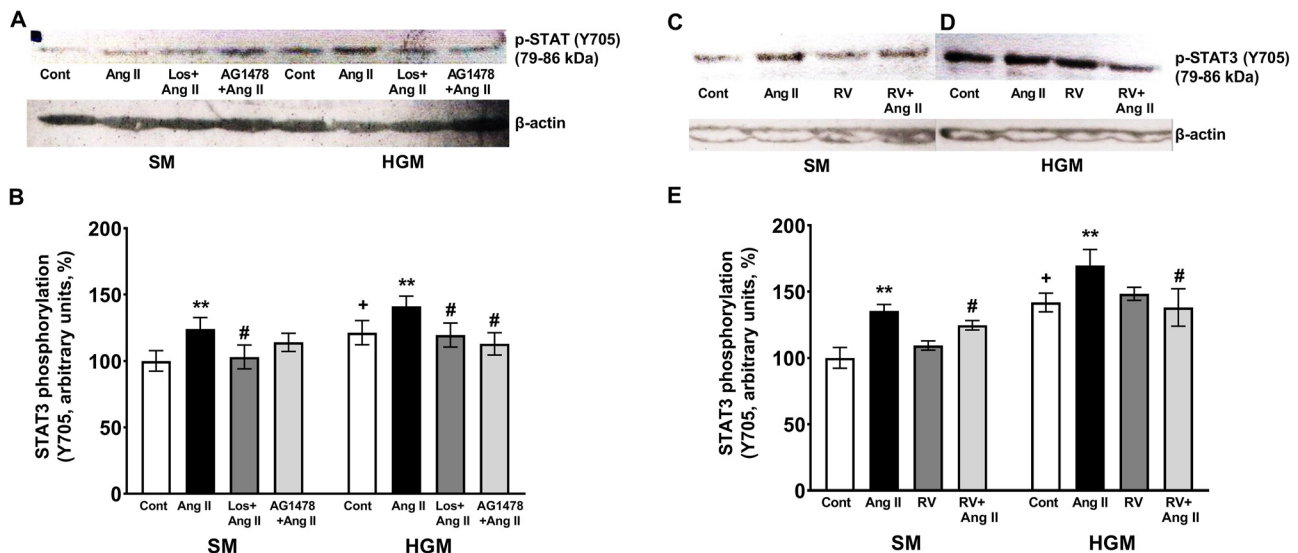


Figure 3: STAT-3 phosphorylation status in VSMCs induced by Ang II. The cells were pre-incubated with the inhibitors at least 30 min before the stimulation of Ang II (100 nM, 5 min). (A, B) The effects of losartan and AG1478 on Ang II-induced STAT3 phosphorylation (Y705). (C, E) The effect of resveratrol (50 μ M) on Ang II-induced STAT3 phosphorylation. Data are expressed as means \pm SEM, $n=3$. **: Significant compared to the control group with $p \leq 0.01$, and # and ##: significant against Ang II with $p \leq 0.05$ and $p \leq 0.01$, respectively. Los, losartan; PD, PD98059; RV, resveratrol; SM, standard media; HGM, high glucose media.

Resveratrol's mechanisms of action involve activating sirtuins and AMPK and modulating various signalling pathways, including MAP kinases and PI3K/Akt [25]. Resveratrol has been shown to impair the Ang II-AT1R axis in ageing mice, downregulating AT1R and Ang II expression

[27]. Our study reveals that resveratrol reduces Akt and ERK1/2 phosphorylation, suggesting its potential to inhibit these pro-proliferative signalling pathways. However, it is noteworthy that the literature presents conflicting evidence regarding the effects of resveratrol on Akt and ERK1/2

phosphorylation. Some studies report inhibition of Akt phosphorylation with resveratrol [16, 28]. Almajdoob et al. found no effect of resveratrol on p-ERK1/2 and p-Akt in Wistar-Kyoto rats, but they observed an impact in spontaneously hypertensive rats [14]. Lin et al. noted an increase in p-Akt levels following resveratrol treatment, albeit at a concentration of 30 μ M in standard conditions, while the same dose reduced p-Akt below control levels [17]. Discrepancies also exist concerning ERK1/2 phosphorylation [11, 14, 17]. For instance, 10 μ M of resveratrol did not affect p-ERK1/2, while 30 μ M reduced p-ERK1/2 in standard conditions. Conversely, 10 and 30 μ M of resveratrol in high glucose conditions increased ERK1/2 phosphorylation in Lin et al.'s study [17]. Guo et al. found that 10 μ M of resveratrol mitigated p-ERK1/2 levels in high glucose-treated VSMCs [11]. Despite these variations, our findings support resveratrol's inhibitory effects on Akt and ERK1/2 phosphorylation, likely contributing to its anti-proliferative properties in VSMCs. Resveratrol may exert this effect by activating PTEN and PP2A [29], inhibiting EGFR transactivation and downstream signalling induced by Ang II [30, 31].

Angiotensin II induces ROS production primarily by activating NADPH oxidases [32]. Elevated ROS levels can activate the ERK1/2 and Akt phosphorylation pathways [33]. Losartan, used in treating hypertension and diabetes, mitigates ROS levels by inhibiting NADPH oxidase activity and exhibits antioxidant properties [34–36]. In contrast, resveratrol, known for its robust antioxidant activity and anti-inflammatory properties, inhibits ROS-induced ERK1/2 and Akt phosphorylation [37]. Additionally, resveratrol upregulates Nrf2 expression, reducing ROS [38, 39]. Compared to losartan, resveratrol's superior inhibitory effect on p-ERK1/2 and p-Akt could stem from its potent antioxidant activity, as resveratrol actively scavenges radicals [34, 37].

We subsequently examined the effects of Ang II and resveratrol on STAT3 phosphorylation, alongside p-Akt and p-ERK1/2, in VSMCs. STAT3 is a transcription factor involved in various cellular processes, including proliferation and inflammation [26]. Our results demonstrate that Ang II increased STAT3 phosphorylation in both standard and high glucose conditions. This elevated STAT3 phosphorylation was effectively countered by resveratrol, losartan, and AG1478, aligning with other researchers' findings [26], highlighting the roles of AT1R [40] and EGFR transactivation [28].

Moreover, pre-treatment with resveratrol significantly reduced STAT3 phosphorylation, suggesting its potential to suppress STAT3-mediated cellular responses. Our findings were in parallel with previous studies showing the suppressive effect of resveratrol on STAT3 phosphorylation

[8, 26]. Resveratrol can succeed in these issues by suppressing upstream and related signalling like Janus kinases (JAKs), Toll-like receptors (TLRs), and Akt [8, 41] and by activating sirtuins [42]. Moreover, resveratrol can induce the suppressor of cytokine signalling proteins (SOCSs), which can inhibit the activation of JAKs [43] and modulate protein tyrosine phosphatases that are responsible for dephosphorylating STAT3 [44]. As mentioned above, PTEN activation and EGFR inhibition can also contribute to resveratrol's ability to inhibit STAT3 phosphorylation.

Resveratrol research primarily originated from cancer studies. Unlike cancer cells, the abnormal proliferation of VSMCs is undesirable as it promotes vascular plaque formation. Therefore, identifying anti-proliferative agents and understanding their mechanisms of action are crucial for treating vascular diseases in the context of cardiovascular diseases. At the cellular level, resveratrol's effectiveness is on par with other specific signalling pathway inhibitors, underscoring its potential.

Furthermore, it is noteworthy that the EGF receptor inhibitor AG1478 exhibits similar efficacy to the Ang II receptor antagonist losartan. It emphasises the significance of EGF receptor transactivation in inducing Ang II-stimulated proliferation and related signalling pathways. The ability of resveratrol to suppress proliferation and associated signalling pathways with a potency akin to PD98059, an inhibitor of the MAPK upstream pathway MEK, deserves emphasis in the context of this study's findings.

However, this study has limitations. It relies on *in vitro* experiments with a limited sample size, and the inhibition was pharmacological. Variability in cellular responses and cell-specific differences may restrict generalizability. Additionally, the focus on specific signalling proteins may overlook other important molecules. Therefore, further validation through animal models or clinical studies is warranted.

Conclusions

In conclusion, our study establishes that Ang II promotes VSMC proliferation and activates ERK1/2, Akt, and STAT3 signalling pathways. Conversely, resveratrol exerts inhibitory effects on VSMC proliferation and suppresses the phosphorylation of Akt, ERK1/2, and STAT3. These findings support the potential therapeutic application of resveratrol in mitigating CVD pathologies associated with VSMC proliferation. Nonetheless, further research is essential to elucidate the mechanisms underlying resveratrol's effects on these signalling pathways and evaluate its efficacy and safety in clinical settings.

Reserach ethics: The research related to animals' use has complied with all the relevant national regulations and institutional policies for the care and use of animals (2019, approval number: 35).

Informed consent: Not applicable.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

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