Journal of Balkan Science and Technology
journal homepage: www.jbst.nku.edu.tr/

Angiotensin II Stimulates Superoxide Anion Generation After Src Phosphorylation in Vascular Smooth Muscle Cells

Fatih UZUNER [1*], Akın YEŞİLKAYA [1]

[1]*Akdeniz University, Faculty of Medicine, Department of Medical Biochemistry, Antalya, TÜRKİYE*

Research Article

Keywords:
Superoxide anion
Hypertension
VSMC
Angiotensin II

Received: 30.10.2023
Accepted: 19.12.2023
Published: 31.12.2023

DOI: 10.55848/jbst.2023.39

ABSTRACT

Angiotensin II (Ang II) causes atherosclerosis and hypertension by damaging the vascular structure. One of the major systems causing hypertension is defined as superoxide anion ($O_2^{\cdot-}$), which is known to have a powerful effect on hypertension progression, produced by NAD(P)H oxidase following Ang II stimulation of vascular smooth muscle cells (VSMCs). Our aim in this study has been to determine whether Src phosphorylation in VSMCs precedes NADPH oxidase activation. Spontaneous hypertensive (SHR) and normotensive Wistar-Kyoto rat (WKY) VSMCs were stimulated by Ang II alone and by Ang II after incubation with AT1R, NAD(P)H oxidase, Src, and PKC inhibitors. The cytochrome c reduction method was employed to measure the AT1R-mediated $O_2^{\cdot-}$ production by NAD(P)H oxidase. The change in Src phosphorylation was detected by the western blot method after the incubation of VSMCs. Following the Ang II stimulation, Src phosphorylation and $O_2^{\cdot-}$ production were found to increase in both groups in comparison with the control group. However, after the Ang II stimulation, Src phosphorylation was reduced with inhibitors other than DPI in both groups. All inhibitors applied after Ang II stimulation suppressed $O_2^{\cdot-}$ formation in both groups. The results suggest that Src and protein kinase C phosphorylation precedes NAD(P)H oxidase activation. In this respect, the ability to control Src phosphorylation in the pathway of NAD(P)H oxidase induction by Ang II emerges as important in drug development and treatment of cardiovascular diseases in hypertensive patients.

1. Introduction

Hypertension is defined as systolic blood pressure values higher than 140 mmHg and/or diastolic blood pressure values above 90 mmHg, where no changes have been made to this recommended classification belonging to the 2003 and 2007 ESH/ESC guidelines [1].

Increased peripheral resistance due to the narrowing of the lumen of resistant vessels is among the major symptoms of hypertension [2]. A small constriction in the vascular lumen may change considerably the vascular resistance. Functional and structural changes may occur in small arteries and arterioles, determining environmental resistance due to hypertension [3]. These changes may manifest themselves as the increased activity of contractile agents, the growth of vascular smooth muscles, increased endothelial function, and vascular inflammation [4].

Remarkably, this condition is associated with increased oxidative stress. Although it has been long known that the oxidation of lipoproteins and low-density lipoproteins causes atherosclerosis, recent studies have shown that early inflammatory events may also cause atherosclerosis [5].

Angiotensin II (Ang II) represents the primary molecule of the renin-angiotensin system which affects the regulation of vascular structure and function. Moreover, Ang II, as a powerful vasoconstrictor, stimulates mitogenic and proinflammatory events. Smooth muscle cell (SMC) growth is stimulated by high Ang II concentrations which increase inflammation and ultimately accelerate (LDL) oxidation [6,7]. The oxidative impacts of Ang II cause various vascular diseases through vascular smooth muscle cell proliferation [8-11]. Ang II displays these effects by stimulating various intracellular signaling steps that increase different reactive oxygen species, especially superoxide anion [12,13]. Ang II activates the NADH/NAD(P)H oxidase enzyme complex and stimulates superoxide anion ($O_2^{\cdot-}$) production [9,11,12].

After having understood that Ang II stimulates NAD(P)H oxidase activation, various studies have been conducted to understand better this pathway. The demonstration that the Src inhibitor PP1/PP2 reduces the formation of Ang II-induced $O_2^{\cdot-}$ suggests that Src phosphorylation is highly important for stimulating NAD(P)H oxidase activation by Ang II [14-17]. Nevertheless, it has been argued that PKC, which activates NAD(P)H oxidase by phosphorylating the p47phox subunit, is an essential molecule in the direct activation of NAD(P)H oxidase by Ang II. Goldblatt was first to reveal the relationship between PKC and $O_2^{\cdot-}$ generation in vessels isolated from 2K1C rat thoracic aorta using the PKC inhibitor chalphostin C [18]. Cai et al. indicated that the main pathway of NAD(P)H oxidase

[*] *Akdeniz University, Faculty of Medicine, Department of Medical Biochemistry, Antalya, TÜRKİYE*
E-mail address: fatihuzuner1979@yahoo.com

activation after Ang II stimulation was to activate the growth factor receptors by C-Src and PKC [19].

Based on these observations, we have investigated whether Src phosphorylation might take place prior to NAD(P)H activation in rat vascular smooth muscle cells (VSMCs). We also examined the change in the amount of $O_2^{-\cdot}$ released between both groups (spontaneous hypertensive rat (SHR) and normotensive Wistar-Kyoto rat (WKY)). Our data shows that NAD(P)H oxidase activation with Ang II stimulation occurrs after the phosphorylation of Src and PKC. We have also determined that $O_2^{-\cdot}$ generation increases in both cell lines stimulated by Ang II. Nevertheless, $O_2^{-\cdot}$ generation is significantly higher in SHR than in WKY.

2. Material and Method

In this research, male rats aged 11-12 weeks were used in two groups as spontaneous hypertensive SHR (Harlan Laboratories, USA) and normotensive Wistar-Kyoto rat (WKY). Rats were kept at a temperature of 23 ± 2 °C with an equal 12-hour light-dark cycle, and no dietary restrictions were applied. The tail-cuff method was employed for recording systolic blood pressure in prewarmed conscious rats [20].

Approval for the experimental protocol was received from the Animal Care and Use Committee of Akdeniz University, and the study was carried out according to the Declaration of Helsinki and the International Association for the Study of Pain (IASP) guidelines.

2.1. Cell Culture

The isolation of VSMCs was performed from the thoracic aorta of SHR and WKY rats, as specified by Günter et al. [21]. Both cell groups were cultured by isolating five normotensive and five spontaneous hypertensive rats from the thoracic aorta (5 WKY and 5 SHR). The cells were cultured until passage 5, and some of them were stored frozen in liquid nitrogen. VSMCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented by 10% fetal bovine serum. VSMCs between passages 6 and 12 were utilized in the experiments and placed in 6-well plates for the experiments (BD Falcon). After reaching the target confluence, the cells were allowed to incubate with serum-free medium for 24 hours before the experiments for immobilization.

2.2. Experimental Protocols

During the experiments, VSMCs in all groups were stimulated by Ang II (100 nmol/L, Sigma) when various pharmacological inhibitors, including Losartan (10 μmol/L, angiotensin type 1 receptor (AT1R) antagonist, Merck Sharp & Dohme), DPI (10 μmol/L, NAD(P)H oxidase inhibitor, Sigma), PP1 (10 μmol/L, Src family tyrosine kinase inhibitor, Calbiochem), and GF109203X (2 μmol/L, PKC inhibitor, Sigma) were either absent or present. The concentrations of the inhibitors used in this study were determined from the previously studied selectivity and sensitivity concentrations [16, 22-27]. In all of the inhibitor studies performed during the research, vascular smooth muscle cells were incubated with inhibitors before the Ang II stimulation.

Protein concentrations were identified with the Pierce Micro BCA protein assay kit (Pierce, Rockford, IL) as indicated in the manual.

2.3. Western Blotting

After stimulation, proteins in lysates from VSMCs were separated by Polyacrylamide Gel Electrophoreses (PAGE) and transferred to a nitrocellulose membrane. The incubation of nitrocellulose membranes was performed at room temperature for a period of 1 hour in a 5% skim milk solution prepared with Tris-buffered saline with the purpose of preventing non-specific binding. After blocking, the membranes were allowed to incubate with the phosphospecific antibody at +4 ^{0}C overnight. Anti-phospho-Src was used as the primary antibody (Tyr416, Upstate, 1:1000). The membranes were incubated in the secondary antibody for one hour at room temperature following overnight incubation with the primary antibody. The signals on the membranes were revealed using chemiluminescence visualized by film autoradiography (PicoWest Super Signal, Pierce, Rockford, IL.). The density of the bands formed on the film was densitometrically quantified by means of Scion Image software (NIH, ScionCorp, Frederick, MD.). After immunoblotting, the membranes were stripped of immune complexes by utilizing a stripping buffer. To normalize the proteins loaded into the wells, the membranes were labeled with b-actin antibody.

2.4. Superoxide Anion Measurement

The cytochrome c reduction method was employed to measure Ang II–stimulated $O_2^{-\cdot}$ generation via NAD(P)H oxidase. Cytochrome c was formed as a result of the reaction of $O_2^{-\cdot}$ with cytochrome c. Cytochrome c is a molecule with the maximum absorbance at 550 nm in the spectrophotometer. We have also determined the $O_2^{-\cdot}$ amount in the medium according to the amount of cytochrome c formed in the reaction [28].

2.5. Data Analysis

Blood pressure measurements were presented as mean ± SD, and statistical analyses were carried out by the Mann-Whitney U test (*$p<0.001$; SHR vs. WKY). Significance was accepted at the $p<0.05$ level.

Passage-matched sets of SHR and WKY VSMCs were used in all experiments. Western blotting data were presented as mean ± SD for a minimum of five experiments. The analysis of blots was carried out by means of Scion Image software, and every blot is representative of a minimum of five experiments. Statistical analyses were performed by the Kruskal-Wallis H test (*$p<0.05$; Ang II vs. other inhibitor applications). Significance was considered at the $p<0.05$ level.

Data on $O_2^{-\cdot}$ production are given as mean ± SD. Statistical analyses were carried out by the Wilcoxon matched-pairs signed-ranks test (*$p<0.05$; Ang II stimulation vs. other inhibitor applications) and the Mann-Whitney U test (# $p<0.05$;

SHR vs. WKY). Significance was considered at the $p<0.05$ level.

3. Results

3.1. Analysis Results

Blood Pressure: The blood pressure of the animals was measured by employing the tail-cuff method prior to the experiments, and the mean values were calculated. Higher blood pressure was determined ($p<0.001$) in SHR (189.6 ± 1.4 mm Hg) in comparison with WKY (132.6 ± 2.8 mm Hg).

The impact of Ang II concentration on Src phosphorylation: Src phosphorylation depended on Ang II concentration. Src phosphorylation was maximum at 1-1000 nM for 5 minutes, and the maximum Src phosphorylation was identified at a 100 nM concentration of Ang II in both groups in this research. We presented the western blot results of the WKY group below (Fig 1).

Ang II-induced time-dependent phosphorylation of Src: Src phosphorylation depends on the time course of Ang II incubation. The maximum responses were obtained during 5 minutes in both groups. Hence we used a 100 nM concentration of Ang II for 5 minutes in this study. We present the western blot results of the WKY group in Fig 2.

During an experiment aimed to review the elevation in Src phosphorylation due to Ang II concentration, it could be observed that the increase in maximum phosphorylation is the result of incubation with 100 nmol/L Ang II for 5 min in VSMCs (Figure 1). As a result of the incubation of VSMCs with 100 nmol/L Ang II at various times, it can be determined that the maximum Src phosphorylation results from 100 nmol/L Ang II stimulation for 5 minutes (Fig 2). For this reason, the subsequent Src experiments were conducted with 100 nmol/L Ang II stimulation for a period of 5 minutes.

Impact of Ang II with/without specific inhibitors on Src phosphorylation: Ang II (100 nM for 5 minutes) stimulation increases Src phosphorylation in both SHR and WKY groups in comparison with controls. On the contrary, priorb to the Ang II stimulation, VSMCs were pre-incubated with losartan (AT1R blocker), DPI (NAD(P)H oxidase inhibitor), PP1 (inhibitor of Src), and GF109203X (inhibitor of PKC). No significant reduction has been revealed in Src phosphorylation in both groups as a result of incubation with DPI, the NAD(P)H oxidase inhibitor, before stimulating VSMCs with Ang II. However, after incubation with inhibitors other than DPI, the Src phosphorylation in VSMCs was significantly reduced in both SHR and WKY groups (Fig 3).

Ang II-induced superoxide anion production: In comparison with the control group (*$p<0.05$), the $O_2^{-\cdot}$ amount in the VSMCs of SHR and WKY rats increased significantly after being stimulated with Ang II. Following the Ang II stimulation, it has been found that the formation superoxide anion increases by approximately 3.6 fold as compared to the control group in WKY group. In the SHR group, the fold rate has been found to be of 3.8. In both SHR and WKY groups, all inhibitors decreased $O_2^{-\cdot}$ generation despite Ang II stimulation (Fig 4).

4. Discussion

Various mechanisms and reasons have been proposed to define hypertension for years. Different vasoconstrictive mechanisms, involving the renin-angiotensin-aldosterone system, endothelin system, vasopressin system, and reactive oxygen radicals (ROS), have been found to play a significant part in developing experimental or human hypertension [29].

The impacts of ROS on the cardiovascular system have recently become an important research subject. Different ROS family members have different effects on cellular functions. In the vascular system, we can list some of the main effects of ROS members, such as cell growth, division, migration, differentiation, and stimulation of many kinases in intracellular signaling pathways. These effects of ROS are often associated with pathologic changes in hypertension [30-33].

Various stimuli affecting the functions of VSMCs, such as cell growth, division, migration, and differentiation, are known, and Ang II is among the important ones. Ang II is indicated by these impacts via NAD(P)H oxidase-derived ROS [31-34].

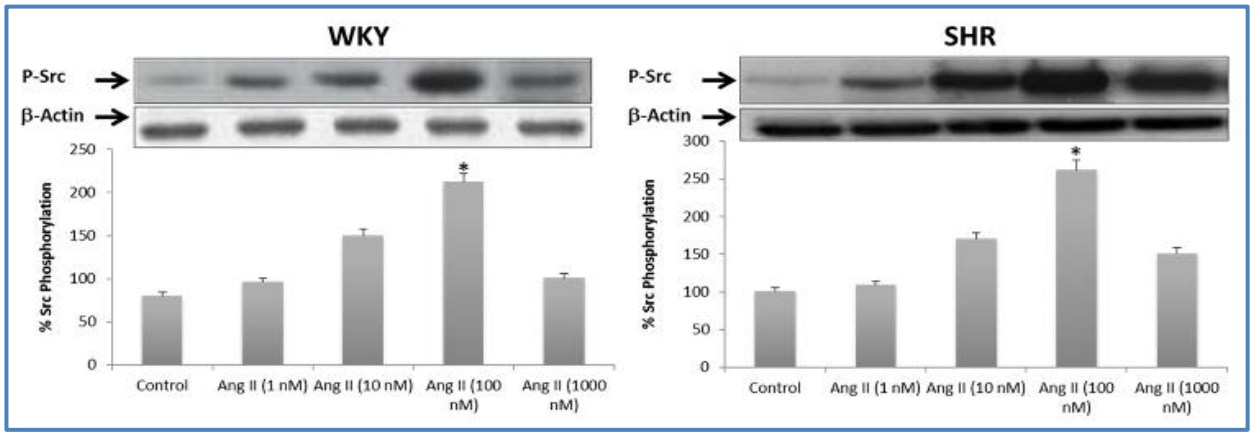

Fig 1. Impact of Ang II concentrations on Src phosphorylation in VSMCs cultured from WKY and SHR rats. VSMCs were incubated with Ang II 1 – 1000 nM for 5 min. The highest Src phosphorylation was detected after 100 nM Ang II incubation in both groups. Bars show the mean ± SD of five experiments. *$P < 0.05$ *vs.* control group.

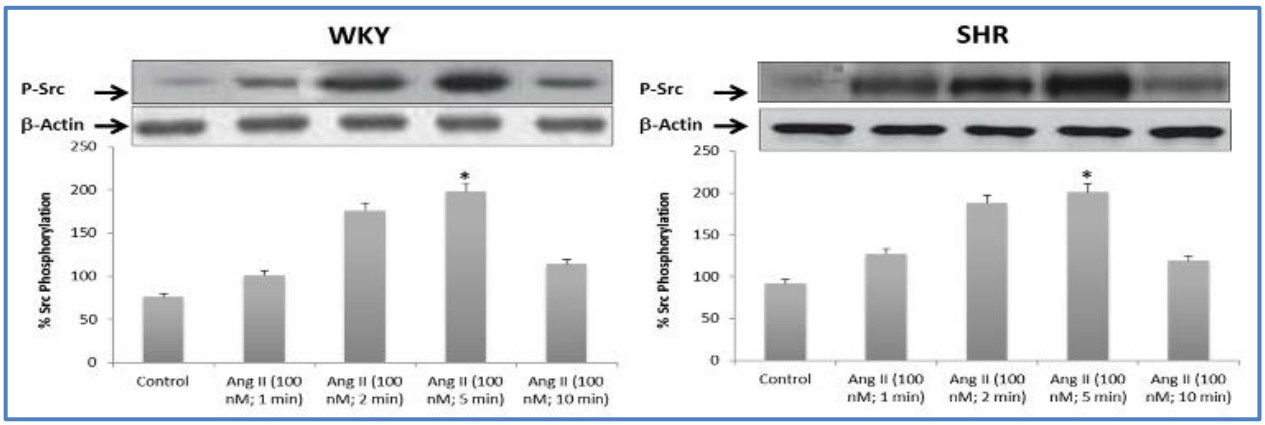

Fig 2. Ang II-induced time-dependent phosphorylation of Src in VSMCs cultured from WKY and SHR rats. VSMCs were incubated with Ang II (100 nM) for 1 to 10 min. Ang II (100nM)-induced phosphorylation of Src was at the maximum level in the VSMCs cultured from both groups for 5 min. Bars show the mean ± SD of five experiments. *P < 0.05 vs. control group.

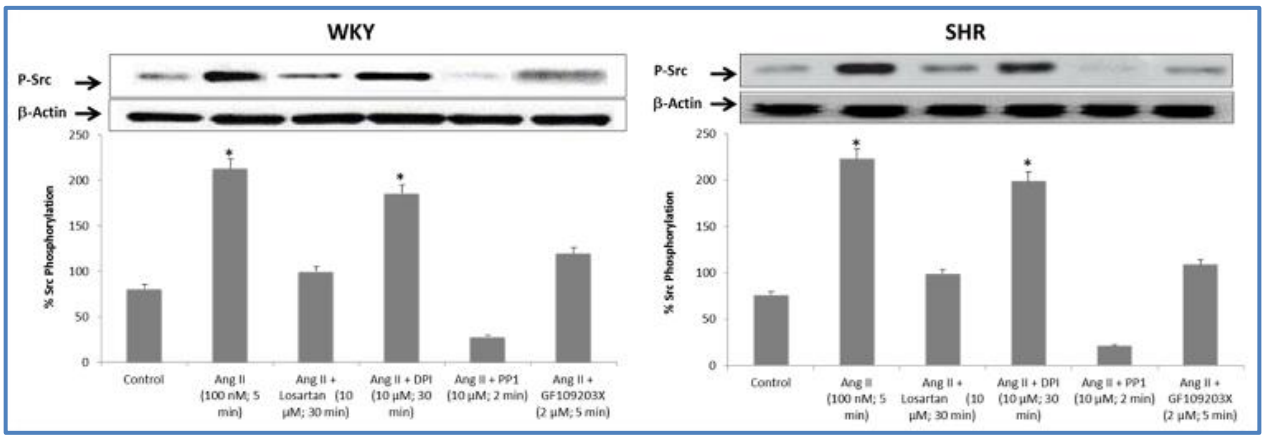

Fig 3. Impacts of inhibitors on the Ang II-induced phosphorylation of Src. Serum-starved VSMCs were pre-incubated with the indicated concentrations of losartan (AT1R blocker), DPI (NAD(P)H oxidase inhibitor), PP1 (Src inhibitor), and GF109203X (PKC inhibitor) for a period of 30 min and stimulated with Ang II for a period of 5 min. Western blot results are shown as averages of five repeated experimental results. The average relative intensities of the bands for Src phosphorylation are shown below the blots. Bars demonstrate the mean ± SD of five experiments. *P < 0.05 vs. control group.

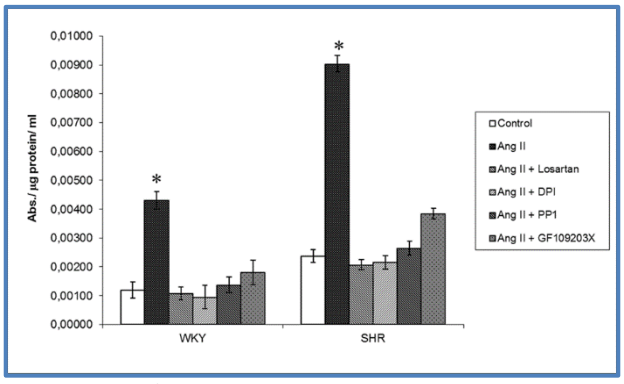

Fig 4. Impacts of inhibitors on Ang II-induced $O_2^{-\cdot}$ generation. Serum-starved VSMCs were pre-incubated with the indicated concentrations of losartan (AT1R blocker), DPI (NAD(P)H oxidase inhibitor), PP1 (Src inhibitor), and GF109203X (PKC inhibitor) for a period of 30 min and stimulated with Ang II for a period of 5 min. Representative results of five experiments are shown. Bars indicate the mean ± SD of five experiments. *P < 0.05 *vs.* control group.

In our study, we suggest that Src upregulation by Ang II can be a significant mediator of elevated $O_2^{-\cdot}$ in VSMCs.

In the present study, we have measured systolic blood pressure in two groups (SHR and WKY) and can argue that the systolic blood pressure of the SHR group is higher in comparison with the WKY group.

According to various researchers, revealing the NAD(P)H oxidase signaling mechanism is very difficult because this signaling mechanism is activated by many stimuli and there are many pathways during this activation approaching each other. The most significant molecule of the NAD(P)H oxidase signaling pathway is probably its major product, $O_2^{-\cdot}$, and the free radicals derived from it. Reactive oxygen species, e.g., $O_2^{-\cdot}$ and H_2O_2, activate tyrosine kinases [35, 36] and are also thought to be associated with the phosphorylation of Src and MAPK [16, 36,37].

Munzel et al. and Lassegue and Griendling have demonstrated that NAD(P)H oxidase organizes cellular functions, including growth, proliferation, and cardiovascular reconstruction in cardiovascular cells [38,39].

Morawietz et al. claim that the renin-angiotensin system is related to cardiovascular disorders while reporting that NAD(P)H oxidase subunits are over-expressed in SRH rats [40].

Zafari et al. have proved that Ang II stimulation increases the amount of hydrogen peroxide (H_2O_2) formed out of NAD(P)H oxidase. The researchers claimed that before Ang II stimulation, cells were incubated with DPI (10 μM; inhibitor of NAD(P)H oxidase), and NAD(P)H oxidase activity was suppressed. Moreover, after the incubation of smooth muscle cells with losartan, it has been demonstrated that the inhibition of H_2O_2 increases along the stimulation of angiotensin [9].

Cruzado et al. indicate that Ang II increases the formation of NAD(P)H-inducible ROS in VSMCs from SHR in hypertension development. The researchers suggest that although the oxidative stress assessed concerning IGF-1R due to Ang II stimulation in VSMCs obtained from SHR is related to an increased blood pressure, this might not be the main event in hypertension pathogenesis [41].

We have previously demonstrated that prior to the stimulation of Ang II cells incubated with losartan and DPI, the amount of superoxide anion is reduced significantly and decreases to basal levels [42].

We have investigated Src phosphorylation in VSMCs, isolated from WKY and SHR. After incubation with the inhibitors and Ang II (100 nM, 5 min) of VSMCs, we found that Src phosphorylation increases in both groups. However, the increase in Src phosphorylation is significantly higher in SHR than in WKY. Nevertheless, before Ang II stimulation, VSMCs were pre-incubated with losartan (AT1R blocker), DPI (NAD(P)H oxidase inhibitor), PP1 (inhibitor of Src), and GF109203X (inhibitor of PKC). In both SHR and WKY groups, no statistically significant difference has been detected between the Src phosphorylation of cells allowed to incubate with inhibitors before Ang II stimulation and the Src phosphorylation of the control group, except for the group incubated with DPI (Figure 3). In this situation, we consider that NAD(P)H oxidase activation occurres after Src phosphorylation in this pathway.

Touyz et al. show that c-Src seriously regulate superoxide anion production via NAD(P)H oxidase due to stimulating p47phox phosphorylation. These new findings describe the NAD(P)H oxidase subunits, especially p47phox, as downstream targets of c-Src [15].

We have revealed that the $O_2^{-\cdot}$ amount increases in two groups after the Ang II stimulation and all inhibitors used in this study suppress the formation of $O_2^{-\cdot}$ anion in both groups.

Sesiah et al. point out that Ang II activates PKC, initiating oxidase activity [16]. Cifuentes et al. claim that oxidase activation after c-Src in the intracellular signal transduction pathway may play an essential part in the selective stimulation of redox-sensitive signaling pathways [43]. Moreover, we have showed that PKC and Src phosphorylation may take place prior to NAD(P)H oxidase activation and found that Ang II induces NAD(P)H oxidase activation via Src.

5. Conclusion

In conclusion, we have shown that Ang II stimulation increases $O_2^{-\cdot}$ generation in VSMCs isolated from hypertensive rats. We have also revealed that this pathway runs through the NAD(P)H oxidase enzyme complex, which is related to the elevated Src phosphorylation following the PKC activation. Based on our data, Src can be identified as an upstream mediator of the intracellular signal transduction pathway regulating Ang II-induced $O_2^{-\cdot}$ generation in VSMCs (Fig 5).

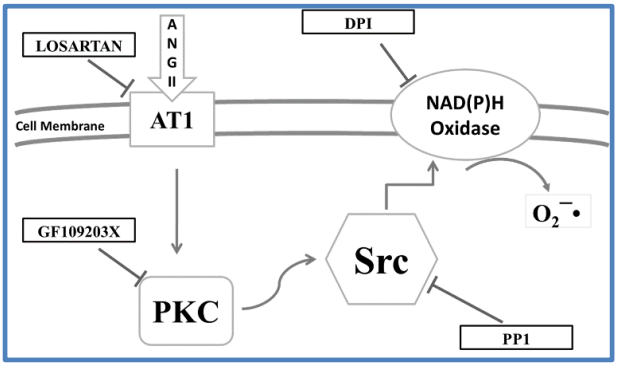

Fig 5. Role of Src in forming O2-. via NAD(P)H oxidase following the stimulation of Ang II in rat VSMCs.

Declaration

Author Contribution: Conceive - F.U.; Design - F.U.; Supervision - A.Y.; Experimental Performance, Data Collection and/or Processing - F.U.; Analysis and/or Interpretation - F.U; Literature Review - F.U.; Writer - F.U.; Critical Reviews - A.Y.

Acknowledgments

The current research was performed as part of an MSc thesis by F. Uzuner presented to the Health Sciences Institute of Akdeniz University.

The present work was supported by a grant obtained from the Research Foundation of Akdeniz University, Turkey (Grant No: 2007.02.0122.009).

Conflict of interests: There is no conflict of interest for all authors.

Orcid-ID

Fatih UZUNER https://orcid.org/0000-0002-5487-4753
Akın YEŞİLKAYA https://orcid.org/0000-0003-0605-4248

References

[1] A. T. F. Members *et al.*, "2013 ESH/ESC Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)," *European Heart Journal*, vol. 34, no. 28, pp. 2159-2219, 2013, doi: 10.1093/eurheartj/eht151.

[2] A. M. Heagerty, E. H. Heerkens, and A. S. Izzard, "Small artery structure and function in hypertension," (in eng), *J Cell Mol Med*, vol. 14, no. 5, pp. 1037-43, May 2010, doi: 10.1111/j.1582-4934.2010.01080.x.

[3] S. Laurent and P. Boutouyrie, "The structural factor of hypertension: large and small artery alterations," (in eng), *Circ Res*, vol. 116, no. 6, pp. 1007-21, Mar 13 2015, doi: 10.1161/circresaha.116.303596.

[4] F. V. Brozovich, C. J. Nicholson, C. V. Degen, Y. Z. Gao, M. Aggarwal, and K. G. Morgan, "Mechanisms of Vascular Smooth Muscle Contraction and the Basis for

Pharmacologic Treatment of Smooth Muscle Disorders," (in eng), *Pharmacol Rev*, vol. 68, no. 2, pp. 476-532, Apr 2016, doi: 10.1124/pr.115.010652.

[5] A. C. Montezano, M. Dulak-Lis, S. Tsiropoulou, A. Harvey, A. M. Briones, and R. M. Touyz, "Oxidative stress and human hypertension: vascular mechanisms, biomarkers, and novel therapies," (in eng), *Can J Cardiol*, vol. 31, no. 5, pp. 631-41, May 2015, doi: 10.1016/j.cjca.2015.02.008.

[6] S. Asgary, A. Sahebkar, M. R. Afshani, M. Keshvari, S. Haghjooyjavanmard, and M. Rafieian-Kopaei, "Clinical evaluation of blood pressure lowering, endothelial function improving, hypolipidemic and anti-inflammatory effects of pomegranate juice in hypertensive subjects," (in eng), *Phytother Res*, vol. 28, no. 2, pp. 193-9, Feb 2014, doi: 10.1002/ptr.4977.

[7] M. Rafieian-Kopaei, M. Setorki, M. Doudi, A. Baradaran, and H. Nasri, "Atherosclerosis: process, indicators, risk factors and new hopes," (in eng), *Int J Prev Med*, vol. 5, no. 8, pp. 927-46, Aug 2014.

[8] M. Yoshizumi, Y. Kyotani, J. Zhao, and K. Nakahira, "Targeting the mitogen-activated protein kinase-mediated vascular smooth muscle cell remodeling by angiotensin II," (in eng), *Ann Transl Med*, vol. 8, no. 5, p. 157, Mar 2020, doi: 10.21037/atm.2019.12.145.

[9] A. M. Zafari *et al.*, "Role of NADH/NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy," (in eng), *Hypertension*, vol. 32, no. 3, pp. 488-95, Sep 1998, doi: 10.1161/01.hyp.32.3.488.

[10] R. M. Touyz, "Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease," (in eng), *Braz J Med Biol Res*, vol. 37, no. 8, pp. 1263-73, Aug 2004, doi: 10.1590/s0100-879x2004000800018.

[11] K. K. Griendling, C. A. Minieri, J. D. Ollerenshaw, and R. W. Alexander, "Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells," (in eng), *Circ Res*, vol. 74, no. 6, pp. 1141-8, Jun 1994, doi: 10.1161/01.res.74.6.1141.

[12] A. Nguyen Dinh Cat, A. C. Montezano, D. Burger, and R. M. Touyz, "Angiotensin II, NADPH oxidase, and redox signaling in the vasculature," (in eng), *Antioxid Redox Signal*, vol. 19, no. 10, pp. 1110-20, Oct 1 2013, doi: 10.1089/ars.2012.4641.

[13] P. K. Mehta and K. K. Griendling, "Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system," (in eng), *Am J Physiol Cell Physiol*, vol. 292, no. 1, pp. C82-97, Jan 2007, doi: 10.1152/ajpcell.00287.2006.

[14] M. A. Laplante and J. de Champlain, "The interrelation of the angiotensin and endothelin systems on the modulation of NAD(P)H oxidase," (in eng), *Can J Physiol Pharmacol*, vol. 84, no. 1, pp. 21-8, Jan 2006, doi: 10.1139/y05-146.

[15] R. M. Touyz, G. Yao, and E. L. Schiffrin, "c-Src induces phosphorylation and translocation of p47phox: role in superoxide generation by angiotensin II in human vascular smooth muscle cells," (in eng), *Arterioscler Thromb Vasc Biol*, vol. 23, no. 6, pp. 981-7, Jun 1 2003, doi: 10.1161/01.Atv.0000069236.27911.68.

[16] P. N. Seshiah, D. S. Weber, P. Rocic, L. Valppu, Y. Taniyama, and K. K. Griendling, "Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators," (in eng), *Circ Res*, vol. 91, no. 5, pp. 406-13, Sep 6 2002, doi: 10.1161/01.res.0000033523.08033.16.

[17] L. L. Camargo *et al.*, "Vascular Nox (NADPH Oxidase) Compartmentalization, Protein Hyperoxidation, and Endoplasmic Reticulum Stress Response in Hypertension," (in eng), *Hypertension*, vol. 72, no. 1, pp. 235-246, Jul 2018, doi: 10.1161/hypertensionaha.118.10824.

[18] T. Heitzer *et al.*, "Increased NAD(P)H oxidase-mediated superoxide production in renovascular hypertension: evidence for an involvement of protein kinase C," (in eng), *Kidney Int*, vol. 55, no. 1, pp. 252-60, Jan 1999, doi: 10.1046/j.1523-1755.1999.00229.x.

[19] H. Cai, K. K. Griendling, and D. G. Harrison, "The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases," (in eng), *Trends Pharmacol Sci*, vol. 24, no. 9, pp. 471-8, Sep 2003, doi: 10.1016/s0165-6147(03)00233-5.

[20] J. Ibrahim, B. C. Berk, and A. D. Hughes, "Comparison of simultaneous measurements of blood pressure by tail-cuff and carotid arterial methods in conscious spontaneously hypertensive and Wistar-Kyoto rats," (in eng), *Clin Exp Hypertens*, vol. 28, no. 1, pp. 57-72, Jan 2006, doi: 10.1080/10641960500386817.

[21] S. Gunther, R. W. Alexander, W. J. Atkinson, and M. A. Gimbrone, Jr., "Functional angiotensin II receptors in cultured vascular smooth muscle cells," (in eng), *J Cell Biol*, vol. 92, no. 2, pp. 289-98, Feb 1982, doi: 10.1083/jcb.92.2.289.

[22] T. M. Paravicini, A. C. Montezano, H. Yusuf, and R. M. Touyz, "Activation of vascular p38MAPK by mechanical stretch is independent of c-Src and NADPH oxidase: influence of hypertension and angiotensin II," (in eng), *J Am Soc Hypertens*, vol. 6, no. 3, pp. 169-78, May-Jun 2012, doi: 10.1016/j.jash.2012.01.002.

[23] M. E. Atef and M. B. Anand-Srivastava, "Enhanced expression of Gqα and PLC-β1 proteins contributes to vascular smooth muscle cell hypertrophy in SHR: role of endogenous angiotensin II and endothelin-1," (in eng), *Am J Physiol Cell Physiol*, vol. 307, no. 1, pp. C97-106, Jul 1 2014, doi: 10.1152/ajpcell.00337.2013.

[24] D. Bokemeyer, U. Schmitz, and H. J. Kramer, "Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor," (in eng), *Kidney Int*, vol. 58, no. 2, pp. 549-58, Aug 2000, doi: 10.1046/j.1523-1755.2000.t01-1-00201.x.

[25] O. H. Öztürk, A. Çetin, A. Tokay, F. Uzuner, G. Tanrıöver, and A. Yeşilkaya, "PDGF-β receptor and PKC have no effect on angiotensin II-induced JAK2 and STAT1 phosphorylation in vascular smooth muscle cells under high

glucose condition," (in eng), *J Recept Signal Transduct Res*, vol. 31, no. 5, pp. 340-9, Oct 2011, doi: 10.3109/10799893.2011.592535.

[26] M. Boumati, Y. Li, and M. B. Anand-Srivastava, "Modulation of ANP-C receptor signaling by arginine-vasopressin in A-10 vascular smooth muscle cells: role of protein kinase C," (in eng), *Arch Biochem Biophys*, vol. 415, no. 2, pp. 193-202, Jul 15 2003, doi: 10.1016/s0003-9861(03)00226-1.

[27] R. M. Touyz and E. L. Schiffrin, "Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D-dependent NAD(P)H oxidase-sensitive pathways," (in eng), *J Hypertens*, vol. 19, no. 7, pp. 1245-54, Jul 2001, doi: 10.1097/00004872-200107000-00009.

[28] S. I. Dikalov, W. Li, P. Mehranpour, S. S. Wang, and A. M. Zafari, "Production of extracellular superoxide by human lymphoblast cell lines: comparison of electron spin resonance techniques and cytochrome C reduction assay," (in eng), *Biochem Pharmacol*, vol. 73, no. 7, pp. 972-80, Apr 1 2007, doi: 10.1016/j.bcp.2006.12.012.

[29] A. C. Montezano and R. M. Touyz, "Reactive oxygen species, vascular Noxs, and hypertension: focus on translational and clinical research," (in eng), *Antioxid Redox Signal*, vol. 20, no. 1, pp. 164-82, Jan 1 2014, doi: 10.1089/ars.2013.5302.

[30] K. K. Griendling, D. Sorescu, B. Lassègue, and M. Ushio-Fukai, "Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology," (in eng), *Arterioscler Thromb Vasc Biol*, vol. 20, no. 10, pp. 2175-83, Oct 2000, doi: 10.1161/01.atv.20.10.2175.

[31] S. Xu and R. M. Touyz, "Reactive oxygen species and vascular remodelling in hypertension: still alive," (in eng), *Can J Cardiol*, vol. 22, no. 11, pp. 947-51, Sep 2006, doi: 10.1016/s0828-282x(06)70314-2.

[32] Q. Chen, Q. Wang, J. Zhu, Q. Xiao, and L. Zhang, "Reactive oxygen species: key regulators in vascular health and diseases," (in eng), *Br J Pharmacol*, vol. 175, no. 8, pp. 1279-1292, Apr 2018, doi: 10.1111/bph.13828.

[33] G. Togliatto, G. Lombardo, and M. F. Brizzi, "The Future Challenge of Reactive Oxygen Species (ROS) in Hypertension: From Bench to Bed Side," (in eng), *Int J Mol Sci*, vol. 18, no. 9, Sep 15 2017, doi: 10.3390/ijms18091988.

[34] T. M. Paravicini and R. M. Touyz, "Redox signaling in hypertension," (in eng), *Cardiovasc Res*, vol. 71, no. 2, pp. 247-58, Jul 15 2006, doi: 10.1016/j.cardiores.2006.05.001.

[35] L. Wu and J. de Champlain, "Superoxide anion-induced formation of inositol phosphates involves tyrosine kinase activation in smooth muscle cells from rat mesenteric artery," (in eng), *Biochem Biophys Res Commun*, vol. 259, no. 2, pp. 239-43, Jun 7 1999, doi: 10.1006/bbrc.1999.0762.

[36] A. Corcoran and T. G. Cotter, "Redox regulation of protein kinases," (in eng), *Febs j*, vol. 280, no. 9, pp. 1944-65, May 2013, doi: 10.1111/febs.12224.

[37] M. A. Laplante, R. Wu, A. El Midaoui, and J. de Champlain, "NAD(P)H oxidase activation by angiotensin II is dependent on p42/44 ERK-MAPK pathway activation in rat's vascular smooth muscle cells," (in eng), *J Hypertens*, vol. 21, no. 5, pp. 927-36, May 2003, doi: 10.1097/00004872-200305000-00017.

[38] T. Münzel, U. Hink, T. Heitzer, and T. Meinertz, "Role for NADPH/NADH oxidase in the modulation of vascular tone," (in eng), *Ann N Y Acad Sci*, vol. 874, pp. 386-400, Jun 30 1999, doi: 10.1111/j.1749-6632.1999.tb09253.x.

[39] B. Lassègue and K. K. Griendling, "NADPH oxidases: functions and pathologies in the vasculature," (in eng), *Arterioscler Thromb Vasc Biol*, vol. 30, no. 4, pp. 653-61, Apr 2010, doi: 10.1161/atvbaha.108.181610.

[40] H. Morawietz, M. Weber, U. Rueckschloss, N. Lauer, A. Hacker, and G. Kojda, "Upregulation of vascular NAD(P)H oxidase subunit gp91phox and impairment of the nitric oxide signal transduction pathway in hypertension," (in eng), *Biochem Biophys Res Commun*, vol. 285, no. 5, pp. 1130-5, Aug 3 2001, doi: 10.1006/bbrc.2001.5312.

[41] M. C. Cruzado, N. R. Risler, R. M. Miatello, G. Yao, E. L. Schiffrin, and R. M. Touyz, "Vascular smooth muscle cell NAD(P)H oxidase activity during the development of hypertension: Effect of angiotensin II and role of insulinlike growth factor-1 receptor transactivation," (in eng), *Am J Hypertens*, vol. 18, no. 1, pp. 81-7, Jan 2005, doi: 10.1016/j.amjhyper.2004.09.001.

[42] F. Akçit, "The effect of Ang II on superoxide anion production via NAD(P)H oxidase enzyme complex in primary cell culture made from rat aorta vascular smooth muscle cells," Medicine, Akdeniz University, 2005.

[43] M. E. Cifuentes and P. J. Pagano, "c-Src and smooth muscle NAD(P)H oxidase: assembling a path to hypertrophy," (in eng), *Arterioscler Thromb Vasc Biol*, vol. 23, no. 6, pp. 919-21, Jun 1 2003, doi: 10.1161/01.Atv.0000077235.97226.82.