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

Fatiha Tabet, Ernesto L. Schiffrin, Glaucia E. Callera, Ying He, Guoying Yao, Arne Östman, Kai Kappert, Nicholas K. Tonks, Rhian M. Touyz

Abstract—Angiotensin II (Ang II) signaling in vascular smooth muscle cells (VSMCs) involves reactive oxygen species (ROS) through unknown mechanisms. We propose that Ang II induces phosphorylation of growth signaling kinases by redox-sensitive regulation of protein tyrosine phosphatases (PTP) in VSMCs and that augmented Ang II signaling in spontaneously hypertensive rats (SHRs) involves oxidation/inactivation and blunted phosphorylation of the PTP, SHP-2. PTP oxidation was assessed by the in-gel PTP method. SHP-2 expression and activity were evaluated by immunoblotting and by a PTP activity assay, respectively. SHP-2 and Nox1 were downregulated by siRNA. Ang II induced oxidation of multiple PTPs, including SHP-2. Basal SHP-2 content was lower in SHRs versus WKY. Ang II increased SHP-2 phosphorylation and activity with blunted responses in SHRs. Ang II—induced SHP-2 effects were inhibited by valsartan (AT_1R blocker), apocynin (NAD(P)H oxidase inhibitor), and Nox1 siRNA. Ang II stimulation increased activation of ERK1/2, p38MAPK, and AKT, with enhanced effects in SHR. SHP-2 knockdown resulted in increased AKT phosphorylation, without effect on ERK1/2 or p38MAPK. Nox1 downregulation attenuated Ang II–mediated AKT activation in SHRs. Hence, Ang II regulates PTP/SHP-2 in VSMCs through AT_1R and Nox1-based NAD(P)H oxidase via two mechanisms, oxidation and phosphorylation. In SHR Ang II–stimulated PTP oxidation/inactivation is enhanced, basal SHP-2 expression is reduced, and Ang II–induced PTP/SHP-2 phosphorylation is blunted. These SHP-2 actions are associated with augmented AKT signaling. We identify a novel redox-sensitive SHP-2–dependent pathway for Ang II in VSMCs. SHP-2 dysregulation by increased Nox1-derived ROS in SHR is associated with altered Ang II–AKT signaling. **(*Circ Res*. 2008;103:149-158.)**

Key Words: redox signaling ■ protein tyrosine phosphatases ■ SHP-2 ■ MAP kinases ■ AKT ■ vascular smooth muscle cells

Reactive oxygen species (ROS), ubiquitous reactive derivatives of O_2 metabolism that include superoxide anion ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2), are produced primarily by nonphagocytic NAD(P)H oxidase (Nox) in the vasculature.[1,2] This enzyme, of which 4 of the 7 isoforms have been detected in rat vascular cells (Nox1, Nox2, Nox4 and Nox5),[3,4] is regulated by vasoactive peptides, including angiotensin II (Ang II).[1–3] In hypertension, increased ROS production leads to vascular smooth muscle cell (VSMC) growth and inflammation through increased activation of c-Src, mitogen-activated protein (MAP) kinases, and PI3K/AKT.[5,6]

ROS influence many signaling events, mainly through oxidative modification of proteins. Of the many redox-sensitive targets, including protein kinases (MEKK1, PKA, CaM-kinases, c-Src), small G proteins (Rac, Rho), ion channels (Ca^{2+}-channels), and transcription factors (AP-1, NF-κB), protein tyrosine phosphatases (PTPs) are particularly important because they possess a conserved 230 amino acid domain with a reactive and redox-regulated thiol-containing cysteine, which when oxidized makes the protein inactive.[9,10] Typically, prooxidative events are correlated with activation of signaling molecules.[11] Paradoxically, many of the direct oxidation events are inhibitory, blocking activity of target molecules. Protein tyrosine phosphatases play a critical role in the reversible phosphorylation of proteins, where they remove phosphates from downstream substrates thereby counterbalancing effects of protein tyrosine kinases (PTK).

In addition to posttranslational modification by oxidation, PTPs are regulated by tyrosine (or serine) phosphorylation,[12]

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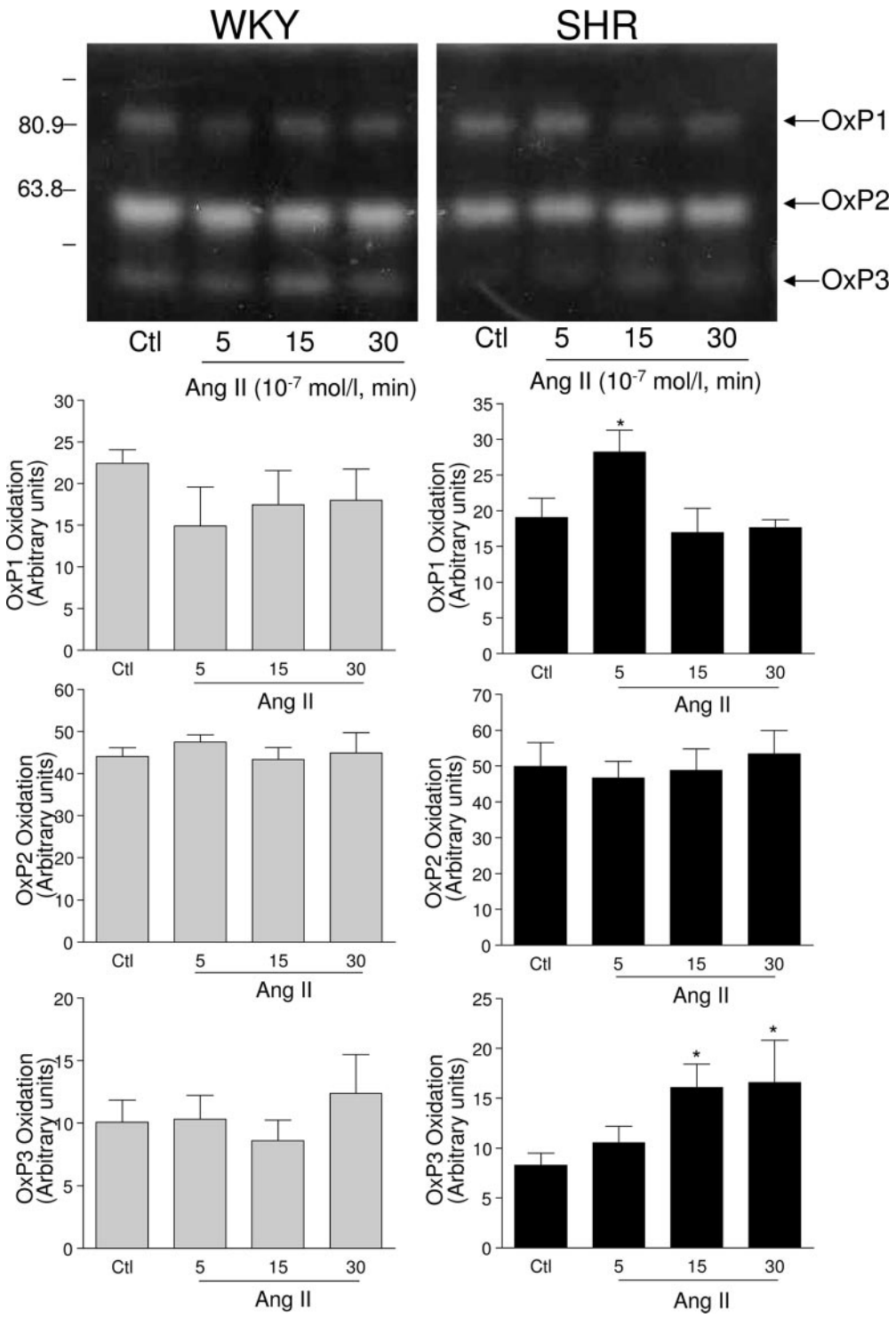

Figure 1. Ang II induces rapid oxidation of multiple PTP in VSMCs. Lysates were prepared under anaerobic conditions in the presence of 10 mmol/L IAA and subjected to the in-gel PTP assay. Top panels represent the in-gel PTP activity assay visualized by autoradiography. Arrowheads indicate the PTPs that were oxidized and arbitrarily called oxidized PTP (OxP)1, OxP2, and OxP3. Bar graphs represent quantification of multiple immunoblots for the indicated PTPs. Data are means±SEM of 3 to 4 experiments. *$P<0.05$ vs control (Ctl).

which renders the protein active. Both thiol oxidation and tyrosine phosphorylation are reversible and dynamic, and both modifications can occur in response to the same stimulus. Whereas tyrosine phosphorylation is usually associated with enzyme activation, oxidation leads to PTP inactivation. Mechanisms of interplay between phosphorylation and oxidation and functional implications of such crosstalk remain unclear, but changes in the local redox environment may fine-tune PTP phosophorylation and consequent downstream signaling.[13,14]

Ang II, through ROS, induces activation of RTKs such as EGFR and PDGFR-β as well as nonreceptor tyrosine kinases,

such as c-Src, which interact with numerous PTPs, including SHP-2, to regulate cell function.[15,16] Activated SHP-2 dephosphorylates substrates that, directly or indirectly, have an inhibitory role in MAP kinase and PI3K/AKT signaling.[17] In cultured VSMCs, oxidative inhibition of PTP is an intrinsic component of H_2O_2 and PDGF-induced signaling, an effect that is reversed by antioxidant treatment.[18] In Chinese hamster ovary (CHO) cells stably expressing a rat vascular angiotensin II type 1A receptor (CHO-AT[1A]), Ang II induced rapid and reversible tyrosine phosphorylation of the PTP SHP-2, which was associated with increased cyclin D1 expression in a PI3K/AKT and MAP kinase-dependent manner.[19]

The exact role of ROS in Ang II–mediated regulation of PTP/SHP-2 and associated kinase signaling in vascular cells remains unclear. We tested the hypothesis that in VSMCs, activation of growth signaling kinases by Ang II involves redox-sensitive regulation of the PTP SHP-2 and that in hypertension enhanced growth signaling is attributable to SHP-2 dysregulation. We also evaluated the role of AT_1R, AT_2R, and NADPH oxidase in these processes. Findings from our study demonstrate that in VSMCs Ang II regulates PTP/SHP-2 through 2 mechanisms, oxidation and phosphorylation. In SHR, signaling through AKT, but not ERK1/2 or p38MAPK, is associated with increased oxidation and blunted phosphorylation of SHP-2. Such events are mediated through AT_1R-regulated Nox1-based NADPH oxidase. These novel data highlight the importance of ROS in oxidation/phosphorylation interplay in PTP/SHP-2 signaling by Ang II in VSMCs.

Materials and Methods

Cell Culture

VSMCs from mesenteric resistance arteries (important in peripheral resistance and in blood pressure regulation) from 16-week-old WKY and SHR rats were isolated and cultured as before described.[7] Low passaged cells rendered quiescent in serum-free medium for 24 hours were used.

In-Gel Phosphatase Assay

The in-gel PTP assay was performed as described by Meng et al.[20] After stimulation with Ang II (10^{-7} mol/L, 0 to 60 minutes), cells were lysed under anaerobic conditions to prevent spontaneous oxidation of PTPs through the air oxygen. Lysates (30 μg) were subjected to an in-gel phosphatase activity assay[21] using SDS-PAGE gels containing [γ-^{32}P]-labeled poly (4:1) Glu-Tyr substrate (1.5×10^6 cpm/20 mL of gel solution, $\approx$2 μmol/L p-Tyr).

Analysis of Oxidized SHP-2 Using the oxPTP Antibody

An oxPTP antibody was used to detect reversible oxidation of PTPs derived from cell extracts. The oxPTP antibody was developed to specifically recognize the sulfonic acid form of catalytic site cysteines of PTPs.[22,23] VSMCs were stimulated with Ang II (10^{-7} mol/L, 5 and 10 minutes) and H_2O_2 (100 μmol/L, 10 minutes) and lysed in a lysis buffer with or without 30 mmol/L IAA. SHP-2 PTP was collected by immunoprecipitation using anti-SHP-2 antibody. Signals were visualized by chemiluminescence.

Western Blot Analysis of SHP-2 Expression and Phosphorylation

VSMCs from WKY and SHR rats were stimulated with Ang II (10^{-7} mol/L, 5 minutes). Expression and phosphorylation of SHP-2 was measured by immunoblot. In some experiments, cells were pretreated with apocynin (NAD(P)H oxidase inhibitor; 10^{-5} mol/L, 3 hours), valsartan (AT_1 receptor [AT_1R] blocker; 10^{-5} mol/L, 30 minutes) or PD123319 (AT_2R blocker; 10^{-5} mol/L, 30 minutes). Expression and phosphorylation of SHP-2 were measured by Western blot.

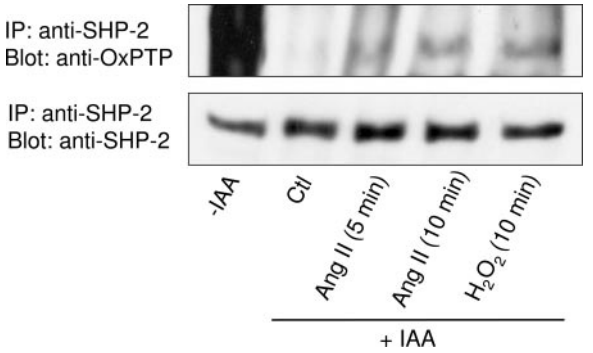

Figure 2. Ang II induces oxidation of SHP-2 in VSMCs. VSMCs from WKY were stimulated with Ang II (10^{-7} mol/L for 5 and 10 minutes) or H_2O_2 (100 μmol/L for 10 minutes) and lysed with or without IAA as indicated. SHP-2 was immunoprecipitated from cell lysate and subjected to pervanadate treatment. Immunoprecipitated SHP-2 was analyzed by consecutive immunoblotting with oxPTP and SHP-2 antibodies.

SHP-2 PTP Activity Assay

VSMCs were stimulated with Ang II (10^{-7} mol/L, 5 minutes) in the absence or presence of the ROS scavenger tiron (10^{-5} mol/L, 30 minutes preincubation) or apocynin (10^{-5} mol/L, 3 hours preincubation) and activity of SHP-2 measured using a PTP activity assay as described.[24]

SHP-2 and Nox 1 siRNA Transfection Studies

VSMCs were transiently transfected with small interfering RNAs (siRNA) against rat SHP-2 and rat Nox 1 (QIAGEN Inc). The siRNA sequences for SHP-2 are available online. Cells were seeded at a density of 2.5×10^5 cells per well in 60-mm plates and exposed to either transfectant alone (HiPerFect Transfection Reagent) or siRNA targeting SHP-2 or Nox 1 as described.[25] A nonsilencing siRNA oligonucleotide sequence (NS siRNA) that does not recognize any known homology to mammalian genes was used as a negative control. After transfection, gene silencing was monitored at the protein levels by immunoblotting using anti–SHP-2 and anti–Nox 1 antibodies. Control cells were exposed to transfectant in the absence of siRNA.

An expanded Materials and Methods section is available online at http://circres.ahajournals.org.

Statistical Analysis

Data are presented as mean$\pm$SEM. Groups were compared using 1-way ANOVA or Student *t* test as appropriate. Newman–Keuls Post test was used to compensate for multiple testing procedures. $P<0.05$ was significant.

Results

Ang II Induces Reversible Oxidation of Multiple PTPs

The reversible oxidation of various PTPs after Ang II stimulation was assessed by a modified in-gel PTP assay. Only activity of those PTPs susceptible to ROS-induced oxidation are recovered and visualized by the appearance of a clear white area of dephosphorylation, surrounding the position of the PTP in the gel on the black background of

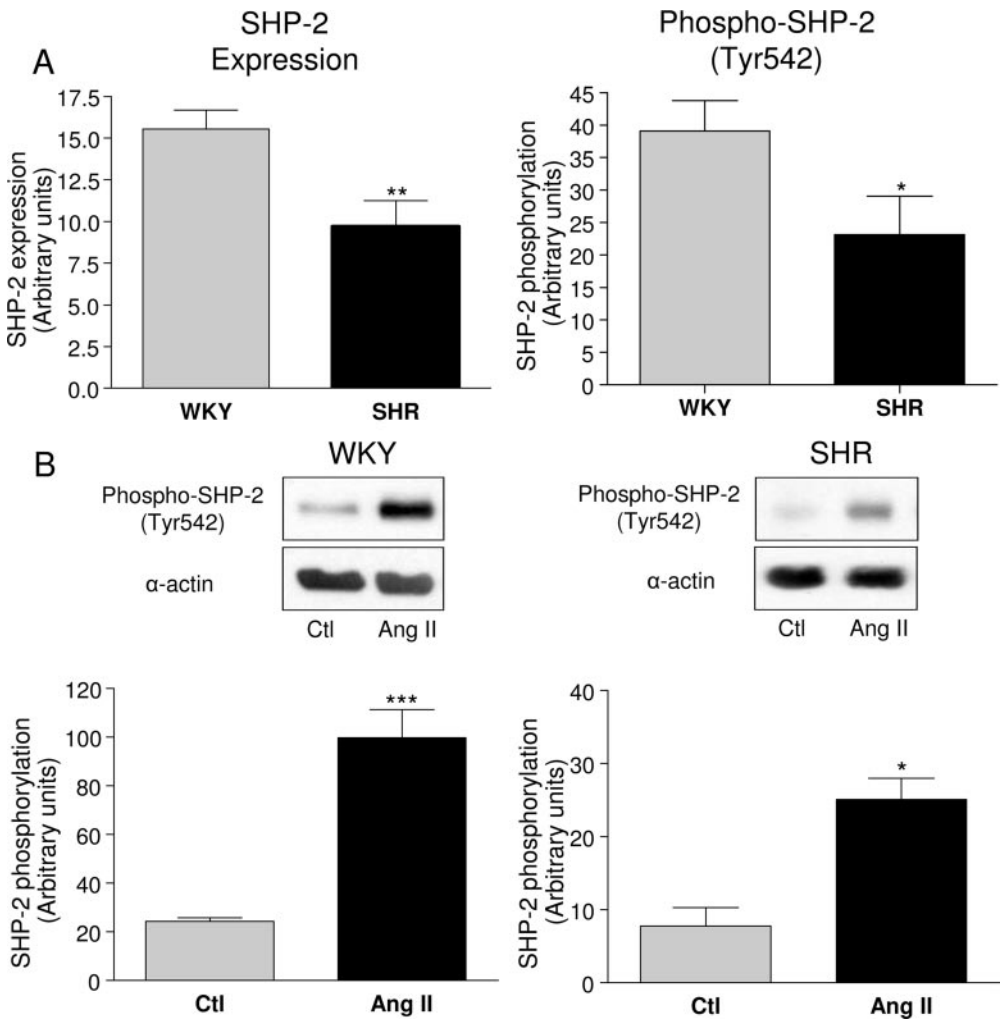

Figure 3. Expression and phosphorylation of SHP-2 in VSMCs from WKY and SHR rats. A, Basal expression and phosphorylation of SHP-2 were determined by Western blotting. B, Cells were stimulated with Ang II (10^{-7} mol/L, 5 minutes) and phosphorylation of SHP-2 was examined by immunoblotting. Membranes were stripped and reprobed with α-actin antibody. Bar graphs represent quantification of multiple immunoblots. Data are presented as means±SEM of 5 to 6 experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs WKY or control (Ctl).

radioactivity labeled substrate. As shown in Figure 1, multiple oxidized PTPs, of varying molecular size, are present in VSMCs. We have arbitrarily named the 3 prominent proteins as oxidized PTP (OxP) 1, OxP2, and OxP3. Ang II (10^{-7} mol/L, 0 to 30 minutes) stimulation resulted in rapid oxidation of multiple PTPs corresponding to OxP1 and OxP3. In SHR cells oxidation of OxP1 and OxP3 was significantly increased ($P<0.05$) after 5 minutes and 15 minutes of Ang II stimulation, respectively. In WKY cells oxidation of OxP3 tended to be increased at 30 minutes. Our results demonstrate the potential for Ang II–mediated ROS generation to induce oxidation and inactivation of PTPs particularly in VSMCs from SHR. As a positive control, VSMCs were also stimulated with H_2O_2, which induced oxidation of multiple PTPs (see supplemental Figure I).

Detection of SHP-2 PTP Oxidation by Ang II Using an oxPTP Antibody

Using a novel antibody-based method,[22] we assessed whether one of the PTPs regulated by Ang II could be SHP-2. This procedure involves alkylation of reduced PTPs with IAA, pervanadate-induced oxidation to sulfonic acid forms of reversibly oxidized PTPs, and detection of oxidized SHP-2 by immunoprecipitation with oxPTP antibody. To increase the efficiency of pervanadate oxidation of reversibly oxidized PTPs, samples were reduced by incubation with DTT. As shown in Figure 2 oxidation of SHP-2 in the basal state is very low. After Ang II stimulation, SHP-2 oxidation was markedly increased. H_2O_2-stimulated (100 μmol/L, 10 minutes) cells were used as a positive control.

Expression and Phosphorylation of SHP-2 in WKY and SHR VSMCs

Lower basal expression ($P<0.01$) and phosphorylation ($P<0.05$) of SHP-2 was observed in SHR cells compared to WKY cells (Figure 3A). Ang II increased SHP-2 phosphorylation in WKY ($P<0.001$) and SHR ($P<0.05$) cells. However, the magnitude of Ang II–induced SHP-2 phosphorylation was lower in cells from SHR versus WKY (Figure 3B).

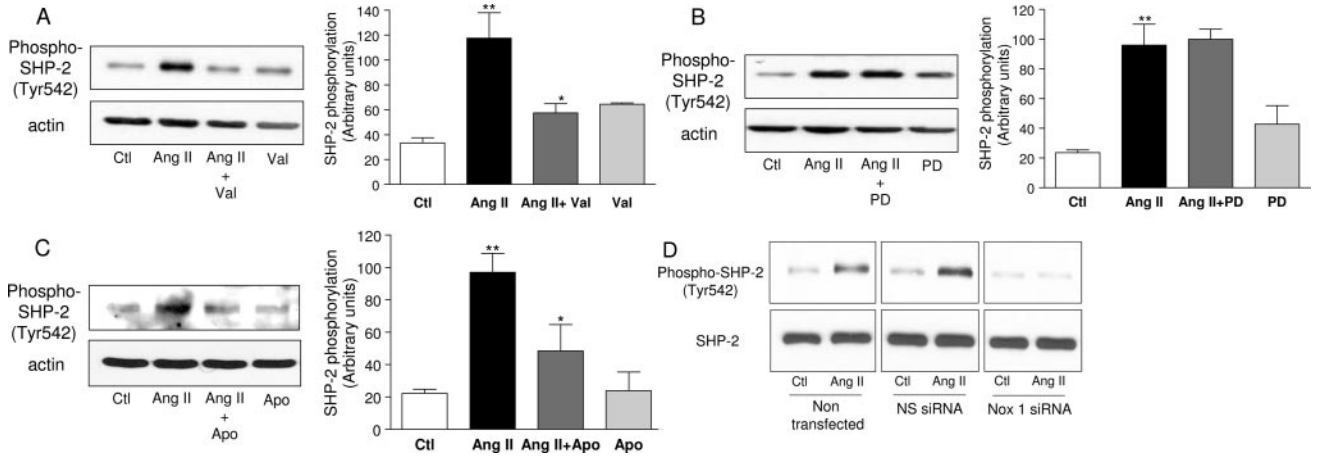

Figure 4. Phosphorylation of SHP-2 by Ang II is mediated through AT_1R and NAD(P)H oxidase. VSMCs from WKY were pretreated with (A) Valsartan (Val, 10^{-5} mol/L) for 30 minutes, (B) PD123319 (PD, 10^{-5} mol/L) for 30 minutes or (C) Apocynin (Apo, 10^{-5} mol/L) for 3 hours and stimulated with Ang II (10^{-7} mol/L, 5 minutes). SHP-2 phosphorylation was determined by Western blot. Total loading was determined by reprobing membranes with α-actin antibody. Left panels are representative Western blots of SHP-2 phosphorylation. Bar graphs represent quantification of multiple immunoblots. Data are presented as means±SEM of 3 to 4 experiments. *$P<0.05$ vs Ang II, **$P<0.01$ vs control (Ctl). D, VSMCs from WKY were transfected with Nox 1 siRNA or nonsilencing (NS) siRNA for 72 hours before stimulation with Ang II (10^{-7} mol/L, 5 minutes). Control cells were exposed to the transfectant without siRNA. SHP-2 phosphorylation was determined by Western blotting. Membranes were stripped and reprobed with SHP-2 antibody.

Ang II Regulation of SHP-2 Is Mediated Through AT_1R and via NAD(P)H Oxidase-Derived ROS

To evaluate possible mechanisms whereby Ang II regulates SHP-2, VSMCs from WKY rats were exposed to the AT_1R blocker valsartan, the AT_2R blocker PD123319, and to the NAD(P)H oxidase inhibitor apocynin, before Ang II stimulation. Ang II–induced phosphorylation of SHP-2 was significantly diminished by preincubation of cells with valsartan ($P<0.05$; Figure 4A) and apocynin ($P<0.05$; Figure 4C). PD123319 did not influence Ang II–induced SHP-2 phosphorylation (Figure 4B). These results suggest that Ang II–dependent regulation of SHP-2 is mediated through AT_1R and via NAD(P)H oxidase-generated ROS. Neither valsartan, PD123319, nor apocynin influenced basal SHP-2 status.

To investigate in further detail whether Nox1 is the NAD(P)H oxidase isoform involved in SHP-2 phosphorylation by Ang II, we transiently downregulated Nox1 expression using siRNA and assessed phosphorylation of SHP-2. As observed in Figure 4D, Ang II–induced phosphorylation of SHP-2 was reduced to basal levels in Nox1 transfected cells compared with control cells (exposed to transfectant without siRNA) and to nonsilencing siRNA transfected cells. These results, together with the apocynin data, suggest that Nox1-based NAD(P)H oxidase-derived ROS plays an important role in SHP-2 regulation by Ang II in VSMCs.

Treatment of VSMCs With Tiron and Apocynin Increases Ang II–Induced SHP-2 PTP Activity in SHR Cells

Using a PTP activity assay to directly measure activity of SHP-2, we investigated whether SHP-2 activity is differentially regulated in SHRs compared with WKY and whether ROS are important in regulating SHP-2 activity. SHP-2 PTP activity was measured in WKY and SHR cells stimulated with Ang II (10^{-7} mol/L, 5 minutes) in the absence or presence of apocynin and the ROS scavenger tiron. Ang II induced a 30% increase in SHP-2 PTP activity in WKY cells only (Figure 5A). Whereas in WKY, apocynin and tiron preincubation decreased Ang II–induced activation of SHP-2 to basal levels (Figure 5A), both apocynin ($P<0.01$) and tiron ($P<0.05$) preincubation increased SHP-2 activity in Ang II–stimulated SHR cells (Figure 5B). These results suggest that in SHR VSMCs, only when ROS bioavailability is reduced is SHP-2 activity increased.

Knockdown of SHP-2 Expression in VSMCs Increases Ang II–Induced Activation of AKT but not of ERK1/2 and p38MAP Kinase

Using RNA interference, we evaluated whether SHP-2 knockdown affects ERK1/2, p38 MAP kinase, and AKT activation by Ang II in VSMCs. As shown in Figure 6, SHP-2 suppression was observed after 24 hours, 48 hours, and 72 hours of incubation with siRNAs. Because the knockdown of SHP-2 was efficient after 24 hours ($P<0.001$), we used this time point for further experiments. The functional significance of SHP-2 oxidation was evaluated by assessing signaling growth responses to Ang II in SHP-2 knockdown cells. We treated cells with Ang II and analyzed the activation of downstream signaling pathways after downregulating SHP-2 with SHP-2 siRNA (Figure 7). Short-term (5 minutes) exposure to Ang II (10^{-7} mol/L) induced an increase ($P<0.05$) in ERK1/2 phosphorylation (Figure 7A), p38MAP kinase phosphorylation (Figure 7B), and AKT phosphorylation (Figure 7C). Using RNA interference, knockdown of SHP-2 was associated with a significant increase in Ang II–induced phosphorylation of AKT in WKY and SHR cells but did not affect ERK1/2 and p38MAP kinases phosphorylation. These results suggest that SHP-2 negatively regulates Ang II activation of AKT pathways in VSMCs. ERK1/2, p38MAP kinase, and AKT phosphorylation were similar in control, siRNA-transfected, and NS siRNA-transfected cells. Protein levels of α-actin were unaffected by siRNA transfection.

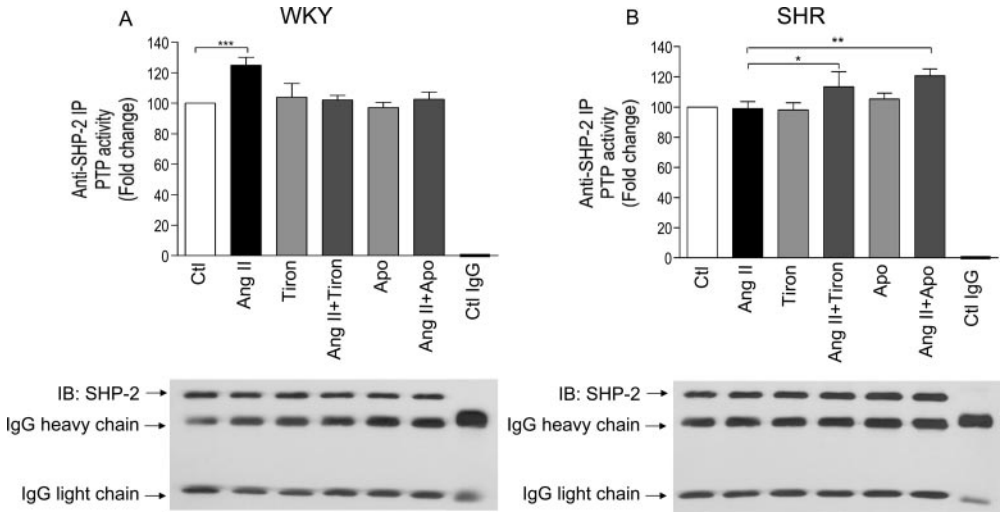

Figure 5. Treatment of cells with tiron and apocynin increases Ang II–induced SHP-2 PTP activity in SHR cells. WKY (A) and SHR (B) VSMCs were stimulated with Ang II (10^{-7}mol/L, 5 minutes) in the presence or absence of tiron (10^{-5} mol/L, 30 minutes preincubation) or apocynin (10^{-5} mol/L, 3 hours preincubation). SHP-2 was immunoprecipitated with anti–SHP-2 antibody. The immune complexes were subjected to 20 mmol/L pNPP and the PTP activity was determined by measuring the absorbance of the supernatant at 410 nm. Bar graphs represent quantification of multiple PTP assays; bottom blots show endogenous SHP-2 immunoprecipitated by the antibody. *$P<0.05$, **$P<0.01$ vs Ang II and ***$P<0.001$ vs control (Ctl).

Knockdown of Nox 1 Expression in VSMCs Decreases Ang II–Induced Activation of AKT in SHR Cells

VSMCs were transfected with Nox1 siRNA for 24 hours, 48 hours, and 72 hours. Maximal downregulation of Nox1 expression was observed after 72 hours of transfection (Figure 8A). To investigate whether knockdown of Nox1 expression by RNA interference decreases Ang II–induced activation of AKT, SHR and WKY VSMCs were stimulated with Ang II for 5 and 10 minutes and AKT phosphorylation was measured in nontransfected cells, in NS siRNA-transfected cells, and in Nox1 siRNA-transfected cells. Ang II induced an increase in AKT phosphorylation by 127% in WKY cells versus 161% in SHR cells (Figure 8B and 8C). Nox 1 siRNA-transfected SHR VSMCs but not WKY cells showed a significant decrease in Ang II–induced phosphorylation of AKT after 5 minutes ($P<0.01$) and 10 minutes ($P<0.05$) of stimulation.

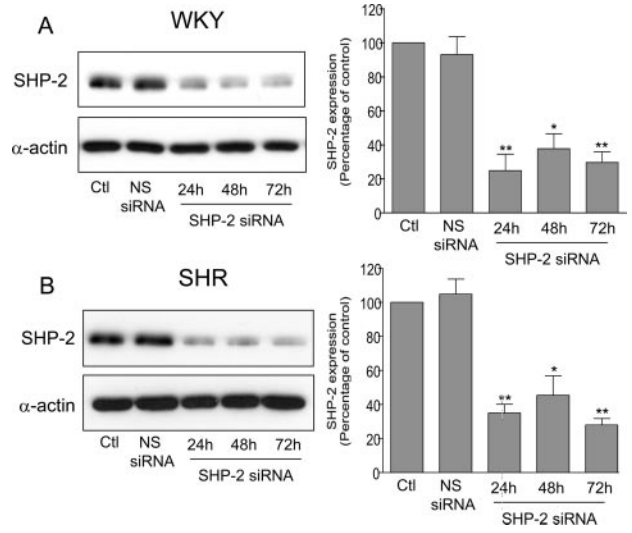

Figure 6. SHP-2 siRNA downregulates SHP-2 expression in WKY (A) and SHR VSMCs (B). VSMCs were transfected with SHP-2 siRNA (24, 48, and 72 hours) or nonsilencing (NS) siRNA (72 hours) and SHP-2 protein expression was assessed by immunoblotting. Control cells were exposed to transfectant without siRNA. Membranes were stripped and reprobed with α-actin antibody. Data are expressed as SHP-2:α-actin ratio and expressed as mean±SEM of 3 experiments. *$P<0.01$ and **$P<0.001$ vs control (Ctl).

Discussion

Major findings from the present study demonstrate that (1) Ang II regulates PTP/SHP-2 in VSMCs through 2 redox-sensitive mechanisms, oxidation and phosphorylation; (2) Ang II–induced activation of SHP-2 is mediated via AT_1R and Nox1-based NAD(P)H oxidase-derived ROS; (3) VSMCs from SHR exhibit increased oxidation/inactivation and blunted redox-sensitive phosphorylation/activation of SHP-2; and (4) increased signaling through AKT, but not through ERK1/2 or p38MAP kinase, by Ang II is induced via Nox1-SHP-2-dependent processes. These novel data highlight the importance of ROS in PTP/SHP-2 signaling by Ang II in VSMCs and suggest that enhanced NAD(P)H oxidase–mediated ROS generation in SHR, as we previously reported,[26,27] may contribute to impaired interplay between oxidation/phosphorylation and dysregulation of PTP/SHP-2, which leads to hyperactivation of downstream AKT signaling pathways. This differential regulation may play a role in redox-sensitive vascular remodeling in hypertension.

Ang II is a multifunctional hormone influencing many cellular processes involved in the regulation of vascular function, including proliferation, migration, and inflammation.[28] The pleiotropic vascular effects are mediated in large part through NAD(P)H oxidase-driven generation of ROS, which function as intracellular and intercellular second messengers to modulate downstream signaling molecules, such as PTPs.[29,30] PTPs are a large, structurally diverse family of which SHP-2, a 68-kDa nontransmembrane-type PTP also known as PTP1D, PTP2C, Syp, and SH-PTP2, appears to be critical in regulating cardiovascular cells.[31]

Despite the importance of PTPs in VSMC signaling, there is a paucity of information on its regulation and little is known about mechanisms whereby Ang II influences these

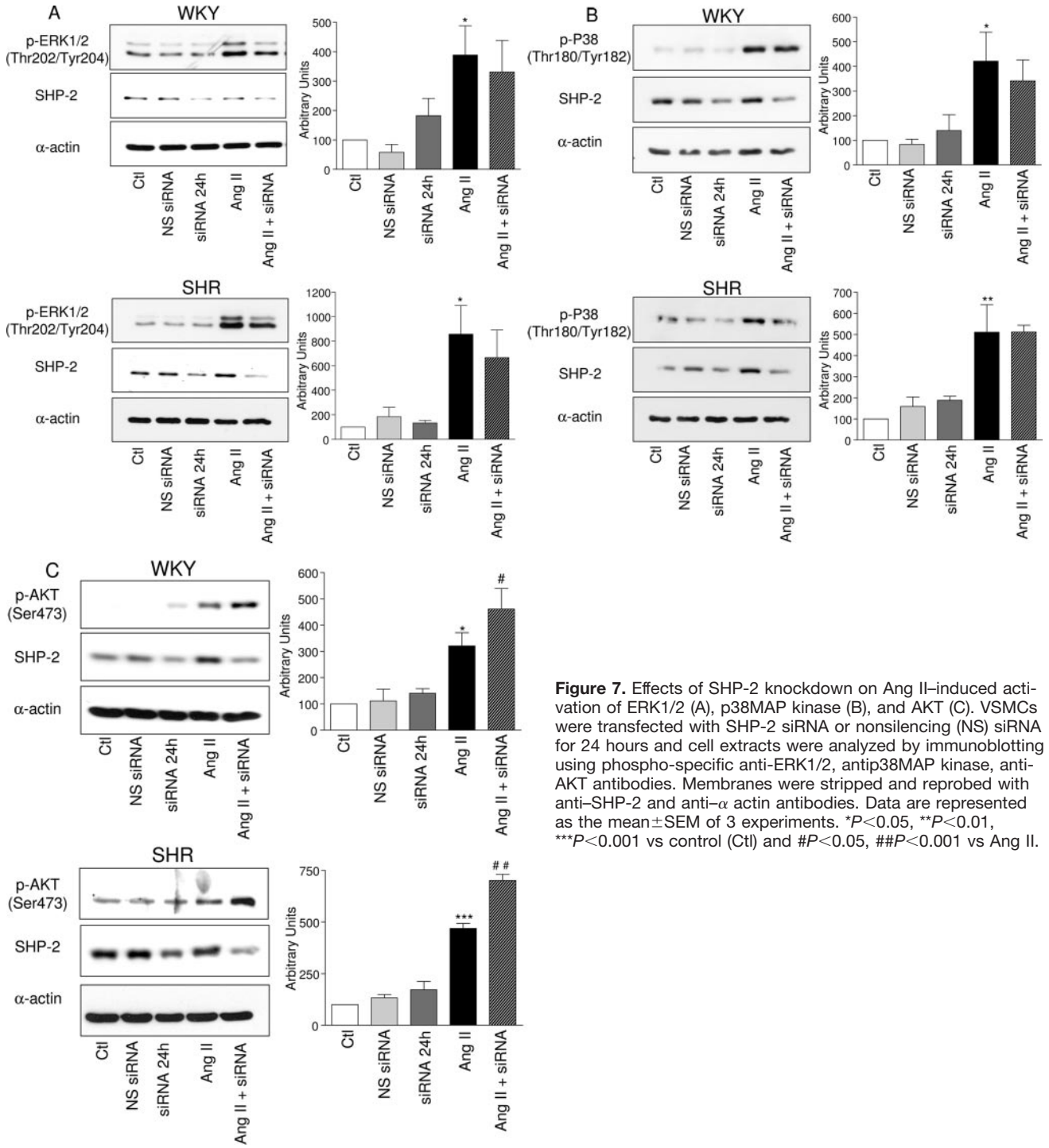

Figure 7. Effects of SHP-2 knockdown on Ang II–induced activation of ERK1/2 (A), p38MAP kinase (B), and AKT (C). VSMCs were transfected with SHP-2 siRNA or nonsilencing (NS) siRNA for 24 hours and cell extracts were analyzed by immunoblotting using phospho-specific anti-ERK1/2, antip38MAP kinase, anti-AKT antibodies. Membranes were stripped and reprobed with anti–SHP-2 and anti–α actin antibodies. Data are represented as the mean±SEM of 3 experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control (Ctl) and #$P<0.05$, ##$P<0.001$ vs Ang II.

phosphatases in VSMCs. Because of their particular structure, PTPs are susceptible to oxidation and consequent inactivation by ROS.[32] Oxidation of PTPs inhibits the nucleophilic property of the PTP which is required for substrate dephosphorylation. Using the in-gel PTP assay we demonstrate that Ang II induces an increase in the reversible inactivation of multiples PTPs of different molecular weights in SHR VSMCs. In support of our findings, others have shown that cell stimulation by PDGFR, EGFR, insulin receptor, and FGFR produces H_2O_2, which reduces PTP activity.[33,34] Lee and colleagues[34] demonstrated that EGF-induced PTP1B inactivation is dependent on reversible oxidation of cysteine residues by H_2O_2. This is particularly important with respect to Ang II, which mediates many of its signaling events in vascular cells through EGFR transactivation.[35] Besides H_2O_2, other ROS may inactivate PTPs by oxidation. Recent studies suggest that PTP1B may be more efficiently regulated by $^{\cdot}O_2^-$ than by H_2O_2.[36]

To further evaluate how Ang II regulates PTPs, we used an antibody-based method to identify a putative PTP that is specifically oxidized by Ang II stimulation in VSMCs. Using this approach, we demonstrate for the first time that Ang

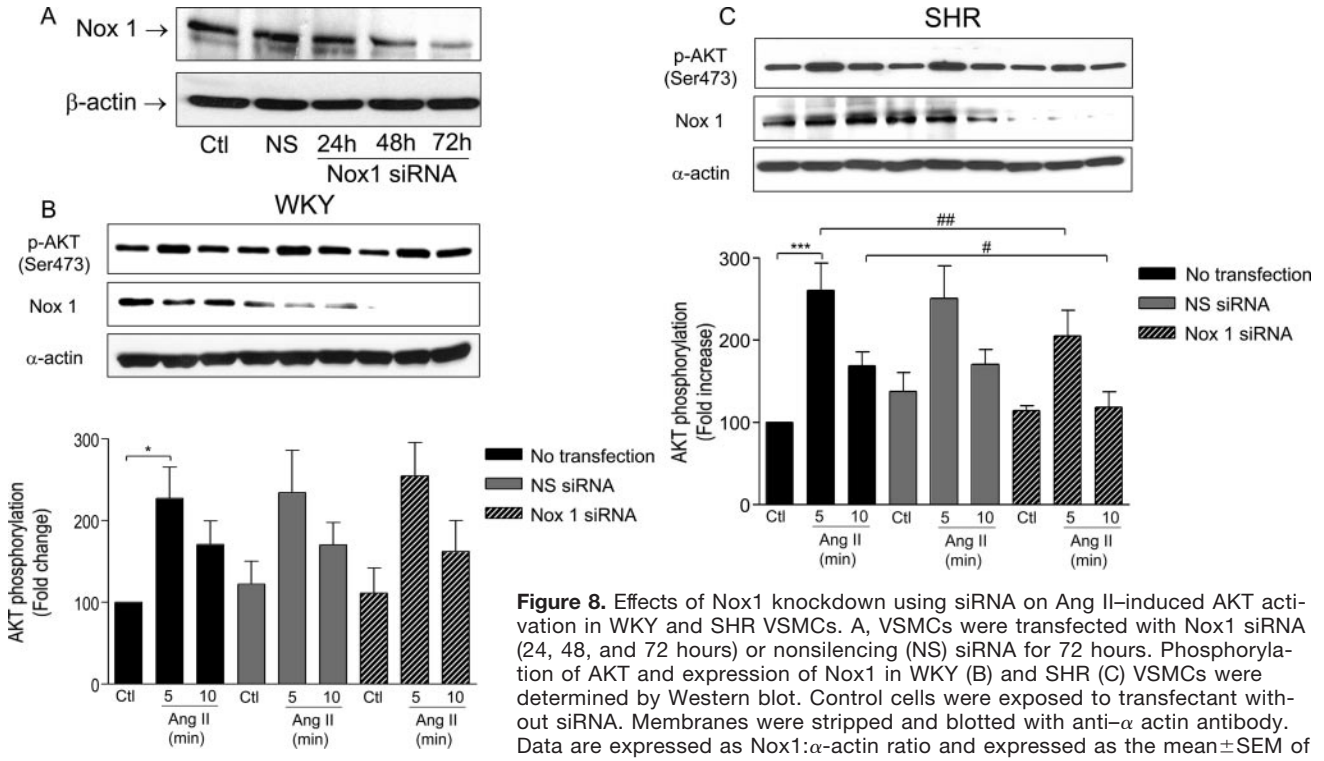

Figure 8. Effects of Nox1 knockdown using siRNA on Ang II–induced AKT activation in WKY and SHR VSMCs. A, VSMCs were transfected with Nox1 siRNA (24, 48, and 72 hours) or nonsilencing (NS) siRNA for 72 hours. Phosphorylation of AKT and expression of Nox1 in WKY (B) and SHR (C) VSMCs were determined by Western blot. Control cells were exposed to transfectant without siRNA. Membranes were stripped and blotted with anti–α actin antibody. Data are expressed as Nox1:α-actin ratio and expressed as the mean±SEM of 4 to 6 independent experiments. *$P<0.05$, ***$P<0.001$ vs control (Ctl); #$P<0.05$ vs Ang II 10 minutes and ##$P<0.01$ Ang II 5 minutes.

II–derived ROS production regulates, by oxidation, SHP-2 PTP (Figure 2). Meng et al[20] previously reported that SHP-2 is reversibly oxidized by other stimuli such as PDGF-BB in RAT-1 cells.

In addition to regulating PTP/SHP-2 by oxidation, we show that VSMC SHP-2 content differs between SHR and WKY and that Ang II stimulation induces significant phosphorylation/activation of SHP-2. In nonstimulated VSMCs the expression and phosphorylation of this tyrosine phosphatase is downregulated in VSMCs from SHRs compared with WKY. Ang II regulates SHP-2 by increasing its phosphorylation in both WKY and SHR cells with a blunted effect in SHR cells. To evaluate mechanisms whereby Ang II influences SHP-2, VSMCs were exposed to the AT_1R blocker, valsartan, the AT_2R blocker PD123319, and the NAD(P)H oxidase inhibitor apocynin before Ang II stimulation. Using these pharmacological tools we show that Ang II–mediated phosphorylation of SHP-2 involves the AT_1R as previously reported[37] and activation of NAD(P)H oxidase. Using siRNA to Nox1, we have extended these findings to demonstrate that the Nox1 isoform of the NAD(P)H oxidase family is involved in Ang II phosphorylation of SHP-2 in VSMCs. Nox1 is present in VSMCs and its expression and activity are increased in hypertension.[38,39]

SHP-2 tyrosine phosphorylation and its phosphatase activity have not been fully characterized in Ang II–mediated redox signaling. In this study, we showed that ROS are important in regulating VSMC SHP-2, particularly in SHR. This is evidenced by our findings that inhibition of NAD(P)H oxidase–derived ROS by apocynin or ROS scavenging by tiron increased Ang II–mediated SHP-2 activity in SHR cells, indicating that when ROS bioavailability is reduced, SHP-2 activity is increased. Downregulation of basal SHP-2 expression and phosphorylation as well as blunted Ang II–induced SHP-2 phosphorylation and activity in SHR may contribute to altered vascular signaling in hypertension.

Exact functions of vascular SHP-2 await elucidation.[40] In stable cell lines overexpressing wild-type SHP-2, SHP-2 dephosphorylated focal adhesion-associated proteins including paxillin, p130Cas, and tensin with associated decreased numbers of focal adhesions within cells.[41] These data indicate a role for SHP-2 in the regulation of the cellular architecture of VSMCs, suggesting that this phosphatase might be instrumental in vascular remodeling.[41] In SHP-2 overexpressing VSMCs, Ang II–induced activation of c-Src and ERK1/2 was abrogated.[42] Here we investigated the functional significance of Ang II–regulated SHP-2 by examining effects of Ang II on downstream growth and cell survival signaling pathways in VSMCs in which SHP-2 was downregulated. Our data demonstrate that SHP-2 knockdown by siRNA significantly increased Ang II–induced phosphorylation of AKT in both WKY and SHR cells, suggesting that SHP-2 negatively regulates Ang II activation of the AKT pathway in VSMCs. Others have shown a positive relationship between SHP-2 and AKT signaling.[43] Reasons for these conflicting data may relate to the fact the SHP-2 may act as a dual modulator of downstream signaling targets.[44] Catalytic activity of SHP-2 is required for insulin-like growth factor (IGF-1)–induced activation of AKT in VSMCs.[43–45] Unlike other studies, which were performed in cell lines overexpressing SHP-2, we could not demonstrate a role for SHP-2 in ERK1/2 regulation. These conflicting data may relate to the fact that we used

VSMCs derived from primary cultures versus cell lines and that in different conditions SHP-2 differentially regulates downstream kinases, as shown by Doan et al,[42] where SHP-2 negatively regulated MAP kinases without effect on Jak2 tyrosine phosphorylation or Fyn catalytic activity. It may also be possible that in our experimental paradigm other PTPs, such as PTPB1 and MAPK phosphatase, regulate ERK1/2 and p38MAP kinase. Taken together it is becoming increasingly apparent that SHP-2 exhibits specific substrate specificity depending on the cell type and the nature of the agonist.

AKT regulates cellular functions such as migration, proliferation, differentiation, and apoptosis and is critically involved in PI3K-mediated cell survival.[46,47] Activation of AKT signaling is sufficient to block cell death induced by apoptotic stimuli, and transduction of dominant negative AKT inhibits growth factor-induced cell survivor.[48] AKT regulates cell cycle and cellular senescence by modulating cell cycle proteins such us E2F, p21, MDM2, and human telomerase reverse transcriptase subunit (hTERT).[49] AKT also enhances protein synthesis through increasing the phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and S6 kinase.[50] With respect to the role of AKT in vascular homeostasis and angiogenesis, results obtained in our study are relevant because they link Ang II/AT_1R/AKT signaling through redox-sensitive SHP-2, which is important in cross-talk with RTK-mediated PI3-K/AKT activation and vascular remodeling.[51] Of significance we found that Ang II–induced phosphorylation of AKT in SHR is dependent on Nox1-induced ROS because AKT phosphorylation was significantly decreased in Nox1 siRNA transfected SHR VSMCs but not WKY cells. Considering that vascular Nox1-derived ROS is increased in hypertension, it may be possible that vascular changes associated with oxidative stress in SHR are linked to dysregulation of redox-sensitive SHP-2 and altered AKT signaling.

In summary, findings from our study demonstrate that Ang II, through redox-sensitive processes, regulates multiple vascular PTPs, among which SHP-2 PTP is important. We identify 2 mechanisms whereby Ang II modulates PTP/SHP-2, by oxidation and by phosphorylation. In SHR, signaling through AKT, but not ERK1/2 or p38MAPK, is associated with increased oxidation and blunted phosphorylation of SHP-2. Such processes are mediated through AT_1R-regulated Nox1-based NADPH oxidase. Collectively our data highlight the importance of ROS in oxidation/phosphorylation interplay in PTP/SHP-2 signaling by Ang II in VSMCs and identify a novel redox-sensitive SHP-2-dependent pathway that regulates AKT signaling in SHR.

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Disclosures

None.

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