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Apocynin-induced vasodilation involves Rho kinase inhibition but not NADPH oxidase inhibition

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Aims The present study was designed to test the hypothesis that NADPH oxidase inhibition with apocynin would lower blood pressure and improve endothelial function in spontaneously hypertensive rats (SHRs). Although apocyin effectively dilated arterial segments *in vitro*, it failed to lower blood pressure or improve endothelial function. Further experiments were performed in normotensive rats and in NADPH oxidase subunit knock-out mice to test if apocynin-induced vasodilation depends on NADPH oxidase inhibition at all.

Methods and results SHRs were treated with apocynin orally or i.v. Arterial pressure was recorded directly. Rat and mouse arterial function was investigated *in vitro* by small vessel wire myography. NADPH oxidase activity was measured in human granulocytes and in rat vascular preparations. Rho kinase activity was determined by Western blot analysis. Apocynin did not reduce arterial pressure acutely in SHR when given at 50, 100, or 150 mg kg^{-1} day^{-1} orally over 1-week intervals or when given i.v. Apocynin potently inhibited granulocyte NADPH oxidase but not vascular NADPH-oxidase-dependent oxygen radical formation unless exogenous peroxidase was added to vascular preparations. Apocynin dilated rat intrarenal and coronary arteries independently of pharmacological interventions that reduce vascular superoxide radical abundance and actions. Aortic rings from p47$phox^{-/-}$ mice were more sensitive to apocynin-induced dilation than wild-type aortic rings. Rho kinase inhibition reduced or prevented the inhibitory effect of apocynin on agonist-induced vasoconstriction and apocynin inhibited the phosphorylation of Rho kinase substrates.

Conclusion Apocynin *per se* does not inhibit vascular NADPH-oxidase-dependent superoxide formation. Its *in vitro* vasodilator actions are not due to NADPH oxidase inhibition but may be explained at least in part by inhibition of Rho kinase activity. The discrepancy between apocynin-induced vasodilation *in vitro* and the failure of apocynin to lower arterial pressure in SHR suggests opposing effects on arterial pressure-regulating systems *in vivo*. Its use as a pharmacological tool to investigate vascular NADPH oxidase should be discontinued.

1. Introduction

Elevated vascular, renal, and neuronal levels of reactive oxygen species (ROS) may contribute to the development of arterial hypertension through pathogenic mechanisms such as endothelial dysfunction,[1] increased renal sodium retention,[2] and increased sympathetic activity.[3] Substances that reduce ROS bioavailability are currently intensively screened for their potential usefulness as antihypertensive agents.[1,4] Spontaneously hypertensive rats (SHRs) show increased vascular[5] and renal[6,7] ROS abundances. Treatment with an antioxidant-fortified diet,[7] the putative superoxide dismutase mimetic tempol,[8] or with extracellular superoxide dismutase gene transfer[5] lowered arterial pressure in SHR.

Both renal and sympathetic mechanisms contribute to the development and maintenance of hypertension in SHR.[9] The effects of the sympathetic nervous system on hypertension in SHR may be partly mediated by kidney. Thus, SHR transplanted with a kidney from neonatally sympathectomized SHR donors had lower blood pressure and lower renal vascular resistance than SHR transplanted with a kidney from untreated SHR donors.[10,11] The mechanisms by which these effects were mediated are currently unknown, but neonatal sympathectomy resulted in decreased renal NADPH oxidase activity in adult SHR.[11] To investigate the potential role of NADPH-oxidase-derived ROS for the development of hypertension, we treated SHR with the NADPH-oxidase inhibitor apocynin from the neonatal period until the age of 8 weeks and subsequently measured arterial pressure. In addition, we assessed endothelial function in renal and coronary arteries.

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Apocynin (acetovanillone) induces vasodilation *in vitro*[12] and has been used in numerous cardiovascular studies[4] to inhibit NADPH-oxidase-dependent superoxide formation *in vitro* and *in vivo*. Apocynin has a high bioavailability when administered orally to rats[13] and it inhibits granulocyte NADPH oxidase by preventing the assembly of the NADPH oxidase subunits p47phox and gp91phox.[4,14] Despite these characteristics, the substance failed to lower blood pressure or to improve endothelial function in SHR, suggesting that its *in vivo* effects may go well beyond the mere NADPH oxidase inhibition.

Prompted by these results, we investigated if apocynin-induced vasodilation *in vitro* can be attributed to NADPH oxidase inhibition by chemically interfering with superoxide formation in vessel preparations from F344 rats and by using vessel preparations from p47phox$^{-/-}$ as well as gp91phox$^{-/-}$ mice. None of these preparations yielded evidence for an important contribution of NADPH oxidase inhibition to the vasodilatory effects of apocynin. Since these results left the mechanisms underlying the vasodilatory effects of apocynin unexplained, we performed additional experiments to test if hyperpolarizing effects, extracellular Ca^{2+}, PKA or PKG-dependent pathways, or the Rho A/Rho kinase pathway were involved.

2. Methods

2.1 Animals

Male and female SHRs to breed first-generation offspring were obtained from Charles River Laboratories (Sulzfeld, Germany). Normotensive F344 rats were obtained from Harlan-Winkelmann (Borchen, Germany). p47phox$^{-/-}$ mice were kindly provided by Dr R.P. Brandes (Department of Cardiovascular Physiology, University of Frankfurt am Main, Germany). C57BL6, C57BL6N, and gp91phox$^{-/-}$ mice originating from the Jackson Laboratory (Bar Harbor, ME, USA) were obtained from the Division of Laboratory Animal Science, University of Greifswald, Germany. Animals were kept in our local animal facility (relative humidity 60%, temperature 22°C, lights on between 06:00 a.m. and 06:00 p.m.). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with the German Animal Protection Act. Permission was obtained from a governmental committee on animal welfare.

2.2 Apocynin treatment and arterial pressure measurements in SHR

To treat neonatal SHR with apocynin, lactating SHR dams were randomized to the treatment (2 mmol/L apocynin via the drinking water) and control group, respectively, on postnatal day 1. Litter size was between three and seven pups and was not manipulated for the experiments. Daily fluid intake was monitored and apocynin dosage was 40 mg kg^{-1} day^{-1} except for the end of the lactation period in which it increased up to 80 mg kg^{-1} day^{-1} in lactating dams due to high fluid intake. After weaning on postnatal day 22, apocynin treatment (40 mg kg^{-1} day^{-1}) via the drinking water was continued in male offspring until the end of postnatal week 8. Thereafter, animals were equipped with radiotelemetric devices (TA11-PAC40, Data Sciences, St. Paul MN, USA) under ether anaesthesia for recording arterial pressure and heart rate as described in detail elsewhere.[10,11] After 1 week of recovery from surgery, arterial pressure was monitored until the end of postnatal week 20.

In addition, three untreated male SHRs were implanted with telemetric devices at 10 weeks of age to investigate the effects of ongoing oral apocynin treatment on arterial pressure. After 1 week of recovery from surgery, baseline arterial pressure was recorded. Thereafter, apocynin was administered in the drinking fluid at doses of 50, 100, and 150 mg kg^{-1} day^{-1} in weekly intervals while arterial pressure recordings were continued.

Further, two SHRs were instrumented with catheters in the right femoral artery and vein at 10 weeks of age as described previously.[10] After 2 days of recovery from surgery, the arterial line was used for blood pressure recordings in conscious unrestrained animals.[10] The venous line was used for intravenous (i.v.) infusions of apocynin. The drug was dissolved in isotonic saline at 10 mmol/L and slowly infused at 100 μL/min in boluses to cumulatively administer 4, 8, 12, and 16 mg kg^{-1}. After completion of each dosing step, arterial pressure was monitored for 15 min until the next dose was administered. This procedure resulted in a total apocynin dose of 40 mg kg^{-1} administered within 75 min.

2.3 Tissue preparation for *in vitro* studies

Human granulocytes were separated from peripheral venous blood (24 mL) obtained from healthy volunteers after they had given informed consent. The investigation conforms with the principles outlined in the Declaration of Helsinki' (*Cardiovascular Research* 1997;35:2–4). Permission was obtained from a local ethics committee. Blood was collected into EDTA-coated tubes and carefully transferred into test tubes (6 mL blood per tube) containing 2.2 mL PBS with 4% dextran and 1% EDTA. Tubes were closed and stored for sedimentation of red blood cells at 37°C for 30 min to obtain a leucocyte-enriched dextran-plasma fraction which was collected and subjected to density gradient centrifugation (310 *g* for 20 min) using Histopaque™-1077 (Sigma, St. Louis, MO, USA). After removal of the supernatant, the tube with the granulocyte-rich pellet was placed on ice and 2 mL of haemolysis buffer (NH_4Cl 155 mmol/L; $KHCO_3$ 10 mmol/L; disodium-EDTA 0.1 mmol/L; pH 7.36) were added. The pellet was carefully agitated and stored for 5 min at 4°C to remove remaining red blood cells. After three times washing with PBS, cells were counted (ABX Micros 60, Axon Lab, Baden Dättwil, Switzerland) and used for measurements of superoxide production. Cell suspensions contained always more than 85% granulocytes. The remaining cells were mainly mononuclear cells and less than 0.1% red blood cells.

Aortic, cardiac, and renal tissues were excised after the animals had been deeply anaesthetized with ether. Dissections of rat and mouse aortic rings, of rat distal second-order renal artery branches (interlobar arteries), and of rat coronary arteries (septal branches) were performed in ice-cold bicarbonate buffered PSS as described in detail previously.[15,16]

2.4 Measurements of NADPH-oxidase-dependent superoxide formation

NADPH-oxidase-dependent superoxide formation in human granulocytes and in rat aortic rings was measured by lucigenin-enhanced chemiluminescence as described in detail previously.[11] Lucigenin concentration was 10 μmol/L. For measurements in aortic rings, NADPH or NADH (both at 3×10^{-4} mol L^{-1}) was added to the reaction mixture while measurements in granulocytes were performed without additional NADPH or NADH. Other sources of superoxide anion formation were inhibited with L-NAME (33 μmol/L), rotenone (50 μmol/L) and allopurinol (100 μmol/L). To verify our findings with lucigenin-enhanced chemiluminescence, we performed additional experiments using the luminol derivative L-012 (500 μmol/L) instead of lucigenin.

2.5 Isolated vessel function

Rat coronary and interlobar artery segments as well as mouse aortic rings were investigated under isometric conditions with a model 410A small vessel wire myograph (Danish Myotechnology, Aarhus, Denmark) as described in detail elsewhere.[15,16] Concentration–response curves were obtained in cumulative fashion. Agonist-induced vasoconstriction is expressed relative to maximum K^+-induced tension, if not indicated otherwise. Agonist-induced vasodilation is expressed

relative to the tension induced by precontraction with either phenylephrine (PE) (renal interlobar arteries) or arg-vasopressin (AVP) (coronary arteries). AVP was preferred in coronary arteries since PE has only weak constrictory effects in these vessels.[16] Care was taken to precontract vessels from different groups to similar tension.[15,16]

2.6 Rho kinase activity

Rho kinase activity was determined by Western blot analysis. Specifically, we determined the ratio between the combined amount of T567-phosporylated ezrin, T564-phosphorylated radixin, and T558-phosphorylated moesin (PERM), and the total amount of ezrin, radixin, and moesin (ERM) (PERM/total ERM ratio). This approach is based on the rationale that ERM threonine residues are phosphorylated by Rho kinase.[17] It has been successfully applied to determine Rho kinase activity in rodent vascular preparations in other studies.[18,19]

Pilot myograph experiments showed that AVP-induced vasoconstriction was inhibited by the Rho kinase inhibitor Y-27632 (3 μmol/L). Apocynin (400 μmol/L) failed to enhance this effect, suggesting that its vasodilatory action may be mediated by Rho kinase inhibition. Aortas dissected from F344 rats were incubated in PSS gassed with carbogen at 37°C[15,16] for 45 min. Three control aortas remained untreated, three aortas were treated with AVP (3×10^{-8} mol/L) for 20 min, and four aortas were preincubated with apocynin (400 μmol/L) for 15 min before AVP (3×10^{-8} mol/L) was added for another 20 min. Experiments were performed in random order. After incubation, aortas were snap-frozen in liquid nitrogen.

Aortic tissue (150–180 mg) was homogenized in lysis buffer (pH 7.4) containing 25 mmol/L sucrose, 50 mmol/L morpholinepropanesulfonic acid (MOPS), 2 mmol/L ethylene diamine tetraacetate (EDTA), 2 mmol/L ethylene glycol-bis-aminoethylether tetraacetate (EGTA), 5 μL/mL protease inhibitor cocktail, 50 mmol/L sodium fluoride, 20 mmol/L sodium pyrophosphate, 1 mmol/L p-nitrophenyl phosphate, and 40 nmol/L okadaic acid. The homogenate was centrifuged at 500 *g* (10 min, 4°C) and the supernatant was stored at −70°C until Western blot analysis. Protein concentration was determined by the Biuret reaction (Carl Roth, Karlsruhe, Germany).

Equal amounts of aortic protein (10 μg) were electrophoretically separated on 10% SDS polyacrylamide gel with 5% stacking gel and transferred to nitrocellulose. Membranes were blocked with non-fat dry milk and incubated with polyclonal rabbit anti-ERM antibody (1:1000) and polyclonal rabbit anti-PERM antibody (1:1000, Chemicon, Temecula, Canada) followed by incubation with peroxidase-labelled secondary antibodies (goat anti-rabbit, 1:10000, Chemicon, Temecula, Canada). Immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL Plus, Amersham Pharmacia Biotech, Buckinghamshire, UK). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control.

2.7 Drugs and chemicals

Apocynin and the radical scavenger tiron were from Fluka/Riedel-deHaën, Buchs, Switzerland. The NADPH oxidase inhibitor VAS2870[20] was kindly provided by Vasopharm Biotech, Würzburg, Germany. L-012 was obtained from Wako Pure Chemical Industries, Tokyo, Japan. PKA inhibitor Rp-8-Br-cAMPS and PKG inhibitor Rp-8-Br-cGMPS were from Biolog Life Science Institute, Bremen, Germany. Rho kinase inhibitor Y-27632 was from Merck Bioscience, Nottingham, UK. The phosphatase inhibitor okadaic acid was from Biomol Rsearch Laboratoris Inc., Plymouth Meeting, PA, USA. All other drugs and chemicals were from Sigma, Taufkirchen, Germany. All drugs were dissolved in isotonic saline, except for VAS2870 and indomethacin that were dissolved in DMSO. It was confirmed that DMSO at the final concentrations used was without effect on chemiluminescence and myographic measurements.

2.8 Statistics

Data in text, table, and figures are presented as means ± SEM. Sigmoidal curve fitting and parameter calculation of cumulative concentration–response curves were performed with SigmaPlot Version 9.0, SPSS, Chicago, IL. Comparisons of group means were performed with the unpaired *t*-test. Comparisons on group means with repeated measurements including concentration–response curves that did not show a sigmoidal pattern were performed by one- or two-way analysis of variance for repeated measurements, respectively. *Post hoc* testing was performed by the Student–Newman–Keuls test. Differences were taken as significant at $P < 0.05$.

3. Results

3.1 Apocynin does not lower arterial pressure in SHR

Adult SHR that had been treated with apocynin on postnatal days 1–56 had virtually identical arterial pressures as untreated controls (*Figure 1A*). Body weight did not differ significantly between both groups (data not shown).

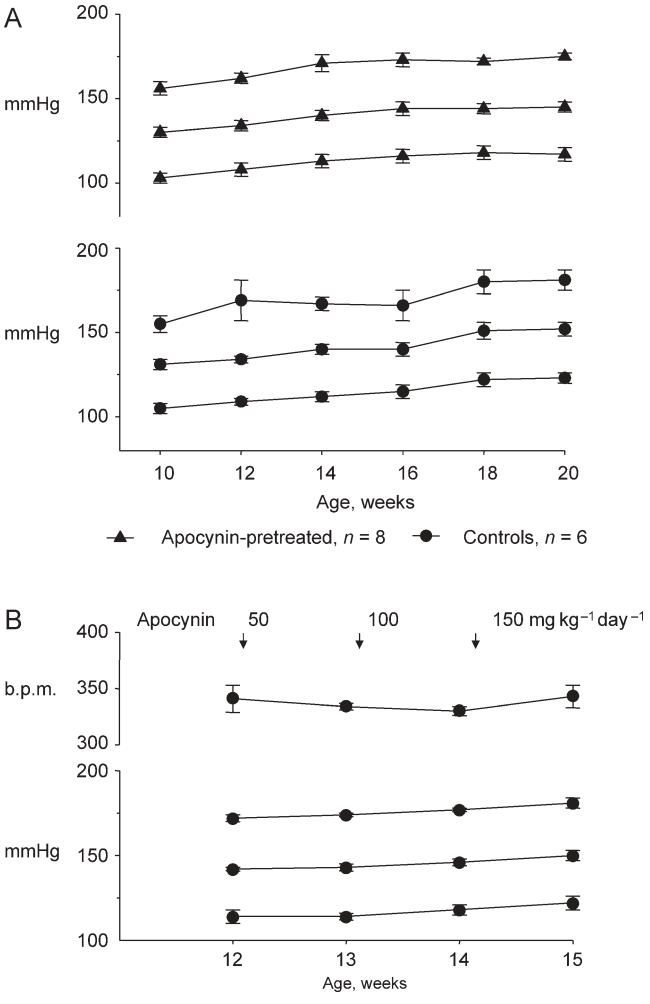

Figure 1 (*A*) Heart rate as well as systolic and diastolic pressure recorded telemetrically between postnatal weeks 10–20 in spontaneously hypertensive rats that had been treated with apocynin from postnatal day 1 until postnatal day 56 and in control rats with spontaneous hypertension. Data represent 12 h mean night-time values from the last recording day of each week. (*B*) Heart rate as well as systolic and diastolic pressure recorded telemetrically in three spontaneously hypertensive rats that were treated with increasing doses of apocynin given in the drinking fluid in three consecutive 1-week intervals.

Apocynin also failed to significantly affect blood pressure when it was given in the drinking fluid at increasing doses over three consecutive 1-week intervals to SHR (*Figure 1B*).

Furthermore, apocynin failed to lower blood pressure when it was given intravenously at increasing doses (final cumulative dose 40 mg kg^{-1}) to two chronically instrumented conscious adult SHR (data not shown). The i.v. administration of apocynin was accompanied by agitation of the animals that started approximately 45 min after the drug had been given and lasted for about half an hour. Although the agitation resolved spontaneously, no further experiments with i.v. administrations of apocynin were performed.

3.2 Early apocynin treatment does not improve endothelial function in adult SHR

Endothelium-dependent, acetylcholine-induced vasodilation was similar between renal interlobar arteries from apocynin-treated and control SHR (*Figure 2A*, left panel). Apocynin-induced vasodilation was also similar between renal interlobar arteries from apocynin-treated and control SHR (*Figure 2A*, right panel). Although L-NAME slightly reversed the apocynin-induced dilation, this effect was incomplete suggesting that the apocynin effects were largely independent of endothelial NO synthesis. Similar results were obtained in coronary arteries from apocynin-treated and control SHR (*Figure 2B*).

3.3 Apocynin-induced vasodilation is not due to NADPH oxidase inhibition

Measurements of NADPH-oxidase-dependent superoxide formation in human granulocytes showed that both apocynin and the NADPH oxidase inhibitor VAS2870 potently blocked NADPH oxidase activity (*Figure 3A*, *Table 1*). Preincubation with tiron (2 mmol/L) and polyethylene glycol (PEG) conjugated superoxide dismutase (SOD) (25 U/mL) effectively reduced the chemiluminescence signals in aortic rings, whereas unconjugated SOD (50 U/mL) was less effective (*Figure 3B*). Neither apocynin nor VAS2870 inhibited the NADPH-oxidase-dependent superoxide production in aortic rings (*Figure 3B* and *C*) or small intrarenal artery segments (not shown) from SHR or F344 rats. Similar results were obtained with NADH instead of NADPH as substrate, but the absolute levels of superoxide formation were one order of magnitude lower (not shown). Data obtained with lucigenin-enhanced chemiluminescence were confirmed with L-012-enhanced chemiluminescence in aortic ring preparations (*Figure 3D*). In the presence of 0.3 U/mL horseradish peroxidase and 10 μmol/L H_2O_2, apocynin potently blocked vascular superoxide formation (*Figure 3E*). Preincubation of interlobar artery segments from F344 rats with 2 mmol/L tiron, 200 μmol/L L-NAME, 200 μmol/L L-NAME plus 3 μmol/L indomethacin, or 1000 U/mL catalase did not significantly affect apocynin-induced vasodilation (data not shown).

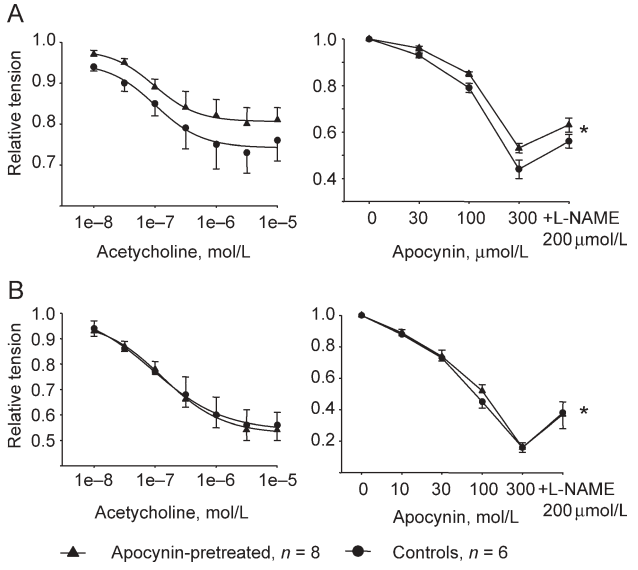

Figure 2 (*A*) Acetylcholine- and apocynin-induced vasodilation in distal interlobar arteries from adult spontaneously hypertensive rats that had undergone postnatal apocynin treatment and from untreated control spontaneously hypertensive rats. L-NAME only partly reversed the apocynin-induced vasodilation. (*B*) Acetylcholine- and apocynin-induced vasodilation in coronary arteries obtained from the interventricular septum of adult spontaneously hypertensive rats that had undergone postnatal apocynin treatment and of untreated control spontaneously hypertensive rats. L-NAME only partly reversed the apocynin-induced vasodilation. *$P < 0.05$ vs. maximum dilation.

To further test whether apocynin-induced vasodilation is mediated by NADPH oxidase inhibition, we preincubated interlobar artery segments from F344 rats with the alternative NADPH oxidase inhibitor VAS2870 (50 μmol/L for 80 min). This treatment resulted in a decreased sensitivity of the vessel segments to PE-induced constriction (*Figure 4A*). In order to achieve similar precontractions in VAS2870-preincubated and control arterial segments we therefore had to apply different doses of PE (*Figure 4B*). Given that precontraction was similar in both groups, there were no statistically significant differences in apocynin-induced vasodilation between VAS2870-preincubated and control vessels (*Figure 4C*). These data suggest that apocynin-induced vasodilation does not depend on the presence of a certain baseline NADPH oxidase activity and may therefore not be due do NADPH oxidase inhibition.

The potential role of NADPH oxidase inhibition for apocynin-induced vasodilation was further investigated in two strains of knock-out mice lacking different NADPH oxidase subunits. Aortic rings from p47$phox^{-/-}$ mice and C57BL6 controls were stretched to 1 mN/mm and active tension in response to 125 mmol/L K^+ was 1.11 ± 0.05 and 0.98 ± 0.05 mN/mm, respectively (n.s.). PE-induced contraction of aortic rings was similar in either mouse strain (*Figure 5A*). Acetylcholine-induced vasodilation was more pronounced in p47$phox^{-/-}$ aortic rings than in controls that showed a biphasic response (*Figure 5B*). Aortic rings from p47$phox^{-/-}$ mice were more sensitive to apocynin (*Figure 5C*) than those from control mice suggesting that NADPH-oxidase derived superoxide antagonizes apocynin-induced vasodilation and indicating that apocynin-induced vasodilation is due to mechanisms different from NADPH-oxidase inhibition. Aortic rings from gp91$phox^{-/-}$ mice were precontracted with potassium (125 mmol/L) because aortic rings from these mice had a higher sensitivity to PE than their controls. Under these conditions apocynin produced similar vasodilation in gp91$phox^{-/-}$ and wild-type controls (not shown). These data indicate in addition that apocynin-induced vasodilation is not due to hyperpolarizing effects.

3.4 Apocynin-induced vasodilation involves the RhoA/Rho kinase pathway

Preincubation of interlobar arteries with apocynin (400 μmol/L) reduced PE-induced contractions in normal PSS and in a

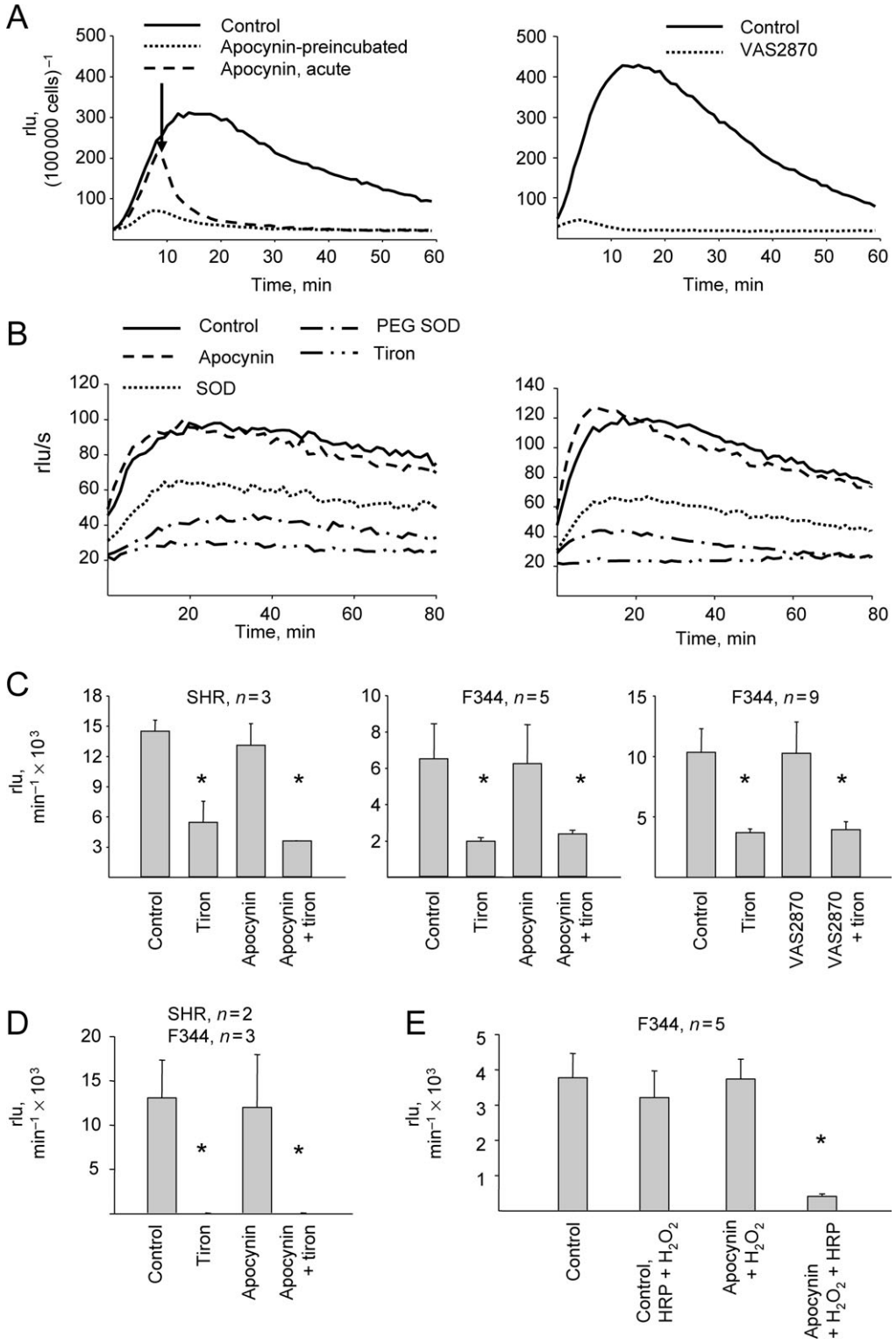

Figure 3 (*A*) Human granulocyte NADPH oxidase activity as measured by lucigenin-enhanced chemiluminescence. Preincubation of granulocytes with apocynin (300 μmol/L) (left panel) or VAS2870 (10 μmol/L) (right panel) effectively inhibited NADPH oxidase activity. Data represent 1 s recording intervals sampled every 60 s. The arrow (left panel) indicates acute addition of apocynin (final concentration 300 μmol/L). (*B*) Time course of NADPH-oxidase-dependent superoxide formation in aortic rings after addition of NADPH. Data represent mean values from two spontaneously hypertensive rats (left) and three F344 (right). Tiron (2 mmol/L), PEG-conjugated superoxide dismutase (25 U/mL), and to a lesser degree unconjugated superoxide dismutase (50 U/mL) reduced the chemiluminescence signal indicating that it was mainly due to superoxide formation. (*C*) Summary of NADPH oxidase activity in aortic rings from spontaneously hypertensive rats and F344, respectively. Apocynin (300 μmol/L) and VAS2870 (10 μmol/L) did not significantly inhibit NADPH-oxidase-dependent superoxide formation (*$P <$ 0.05 vs. control). (*D*) Effect of tiron and apocynin on L-012-enhanced chemiluminescence in aortic rings from spontaneously hypertensive rats and F344 (*$P < 0.05$ vs. control). (*E*) Effects of apocynin on lucigenin-enhanced chemiluminescence in the presence of horseradish peroxidase in aortic ring preparations (*$P < 0.001$). rlu, relative light units.

Table 1 Effects of apocynin and VAS2870 on NADPH-oxidase-mediated superoxide formation of human granulocytes

Superoxide formation, relative light units/(min*100.000 cells)				
Apocynin (300 μmol/L)			VAS2870 (10 μmol/L)	
$n = 5$ individuals			$n = 3$ individuals	
Control	Apocynin	Apocynin, acute	Control	VAS2870
5644 ± 1771	485 ± 218*	1093 ± 303*	3524 ± 1848	80 ± 67*

Freshly prepared granulocytes were incubated with apocynin or VAS2870 at the beginning of the measurements. In additional experiments, apocynin was added 9 min after the measurements were started (apocynin acute, see *Figure 3A*).
*$P < 0.05$ vs. control.

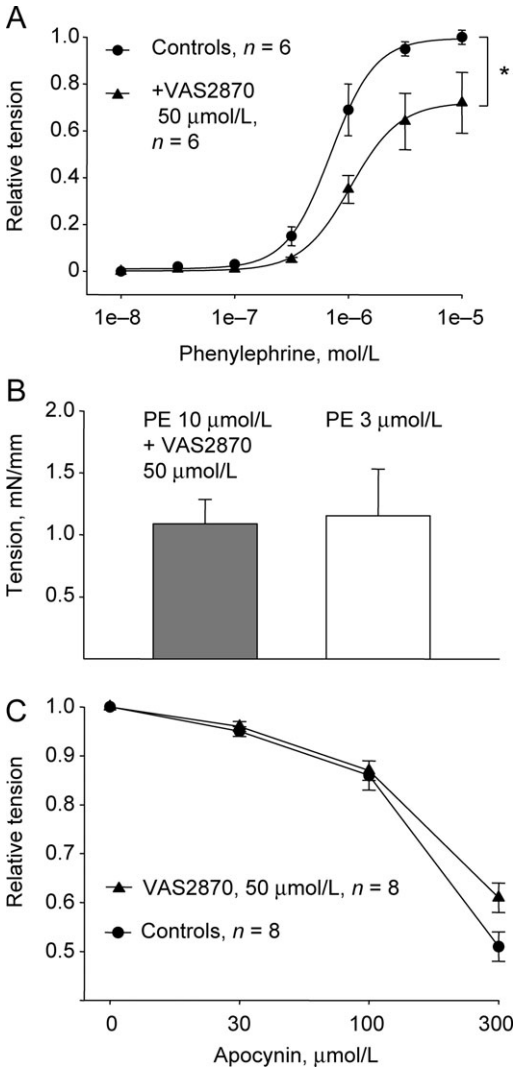

Figure 4 (*A*) Phenylephrine-induced constriction in renal interlobar arteries from F344 rats. Preincubation with VAS2870 significantly reduced phenylephrine-induced vasoconstriction compared with controls that were preincubated with vehicle (DMSO final concentration in the organ bath: 0.1%). (*B*) Absolute active tension in VAS2870- and vehicle-preincubated interlobar arteries precontracted with different concentrations of phenylephrine. (*C*) Apocynin-induced vasodilation in control and VAS2870-pretreated renal interlobar arteries from F344 rats. Vasodilation did not differ significantly between both groups.

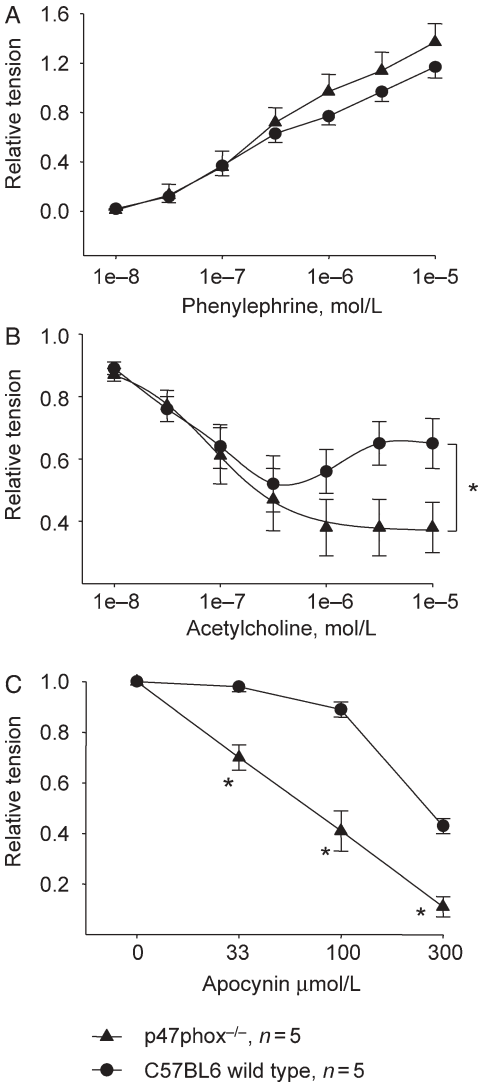

Figure 5 (*A*) Phenylephrine-induced constriction in aortic rings from p47phox$^{-/-}$ and wild-type C57BL6 mice. (*B*) Acetylcholine-induced dilation of aortic rings from p47phox$^{-/-}$ and wild-type C57BL6 mice. (*C*) Apocynin-induced dilation in phenylephrine-precontracted (1 μmol/L) aortic rings. Aortic rings from p47phox$^{-/-}$ were more sensitive to apocynin than aortic rings from wild-type mice. $P < 0.05$ vs. wild-type mice.

Ca^{2+}-free solution containing 1 mmol EGTA (*Figure 6*). PKA inhibition with 250 μmol/L Rp-8-Br-cAMPS or PKG inhibition with 100 μmol/L Rp-8-Br-cGMPS was without significant effects on apocynin-induced vasodilation (not shown). Effective PKA and PKG inhibition was confirmed with control experiments showing potent inhibition of isoproterenol- or Na-nitroprusside-induced vasodilation.

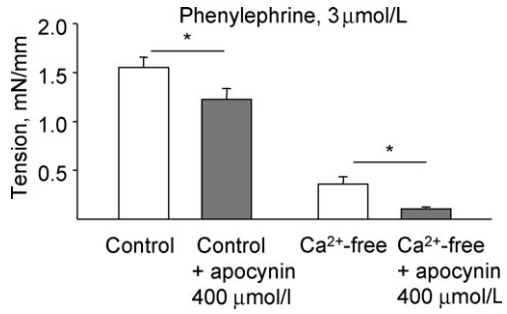

Figure 6 Apocynin effects on maximum phenylephrine-induced tension in renal interlobar arteries from F344 rats. Apocynin significantly reduced phenylephrine-induced tension in physiological saline solution and under Ca^{2+}-free conditions (*$P < 0.05$).

Rho kinase inhibition shifted the PE concentration–response curve to the right and reduced its maximum in interlobar arteries (*Figure 7A*). Addition of apocynin (400 μmol/L) to Y-27632-pretreated vessels did not further shift the PE concentration–response curve to the right but reduced its maximum further (*Figure 7A*). AVP-induced vasoconstriction was more sensitive to Rho kinase inhibition than PE-induced vasoconstriction (*Figure 7B*). Addition of apocynin (400 μmol/L) to Y-27632-pretreated vessels had no statistically significant effects on the AVP concentration–response curve, suggesting that apocynin may require an intact Rho A/Rho kinase pathway to reduce vascular tone. To test if apocynin inhibits Rho kinase activity the ratio of PERM/total ERM was determined by Western blot analysis in F344 rat aortas. AVP alone significantly increased the PERM/total ERM ratio by 30% compared with control aortas, where the ratio of PERM/total ERM did not differ significantly between controls and aortas treated with apocynin and AVP (*Figure 7C* and D).

4. Discussion

In the present study we show that treatment with apocynin did not lower arterial pressure in SHR. There is ample evidence that excess ROS bioavailability contributes to arterial hypertension, including genetic hypertension in SHR.[1–3] Furthermore, it is well established that interventions such as ACE inhibition,[21] AT_1 receptor blockade[22] and sympathetic blockade[10] performed early in life have long-lasting antihypertensive effects in SHR. In renal transplantation experiments kidney grafts from adult SHR donors that had been treated with an ACE inhibitor during a brief period shortly after birth chronically lowered arterial pressure in untreated SHR recipients.[23] Similar results were obtained with neonatally sympathectomized donors.[10] These findings indicate that the kidney contributes importantly to the long-term effects on blood pressure of early antihypertensive interventions. We have recently shown that neonatal sympathectomy is associated with a reduction in renal NADPH oxidase activity in SHR kidneys.[11] To test if NADPH-oxidase-derived ROS plays a critical role in hypertension development in SHR, we subjected rats of this strain to early postnatal treatment with the NADPH oxidase inhibitor apocynin using a dose regimen that has been repeatedly applied in experimental hypertension research.[24–27] In additional experiments we also administered apocynin to adult rats in the drinking fluid or by intravenous infusion. None of these treatments significantly altered arterial pressure in SHR.

These findings are in agreement with one study[26] in SHR but not with another.[27] Unfortunately, both studies[26,27] used tail-cuff measurements to assess arterial pressure. Given the well-known short comings of this technique,[28] the results of these studies[26,27] may be difficult to compare with the present data. Our data are also in agreement with a recent study[25] in rats with endothelin-1-induced hypertension in which blood pressure was measured telemetrically. In this study[25] apocynin also had no significant antihypertensive effect when given at a similar dose (1.5 mmol/L in the drinking fluid) as in our study. In contrast to genetic hypertension in SHR and to endothelin-1-induced hypertension, mineralocorticoid-dependent hypertension may be sensitive to apocynin treatment.[24] The differences in the literature regarding the antihypertensive effects of apocynin may at least in part be explained by different degrees of oxidative stress and inflammation that are associated with different forms of experimental hypertension.

Although apocynin did not lower blood pressure in SHR, it concentration-dependently dilated interlobar and coronary arteries from SHR and normotensive F344 rats. These data are in agreement with previous findings in rat and human blood vessels.[12] We performed several experiments to test whether the vasodilator effects of apocynin are mediated by inhibition of NADPH-oxidase-dependent oxygen radical formation. Apocynin-induced vasodilation could not be significantly inhibited by a radical scavenger such as tiron, with catalase, with VAS2870 or L-NAME suggesting that apocynin-induced vasodilation in interlobar artery segments does not critically depend on NADPH oxidase inhibition or the availability of NO.[12] The latter finding is in agreement with the recent data on renal blood flow in response to apocynin infused into the renal artery.[29]

Our conclusion that apocynin-induced vasodilation does not depend on NADPH oxidase inhibition is strongly corroborated by the results obtained with aortic rings from $p47phox^{-/-}$ and $gp91phox^{-/-}$ mice. In these experiments apocynin had potent vasodilatory effects in aortic ring preparations from both NADPH oxidase subunit-deficient knockout strains. In fact, apocynin-induced vasodilation was significantly stronger in aortic rings from $p47phox^{-/-}$ mice than in aortic rings from wild-type controls, indicating that it was definitely independent of NADPH oxidase inhibition.

The role of NADPH oxidase inhibition as a potential mechanism underlying apocynin-induced vasodilation is further questioned by a recent study[30] reporting that apocynin may not be an inhibitor of vascular NADPH oxidase. The present data agree well with this finding,[30] since apocynin potently inhibited NADPH oxidase activity in granulocytes but not in vascular preparations. The present data do not allow for final conclusions on the NADPH-oxidase-dependent vascular effects of VAS2870.[20]

More than a decade ago, it was shown that the apocynin-induced inhibition of granulocyte NADPH oxidase requires preactivation by the enzyme myeloperoxidase (MPO).[14] Recently,[30] it was demonstrated in transfection experiments using human embryonic kidney cells that MPO activity is also required for apocynin to inhibit NADPH oxidase in cell types other than granulocytes. In agreement with these studies[14,30] our data show that exogenously applied peroxidase can

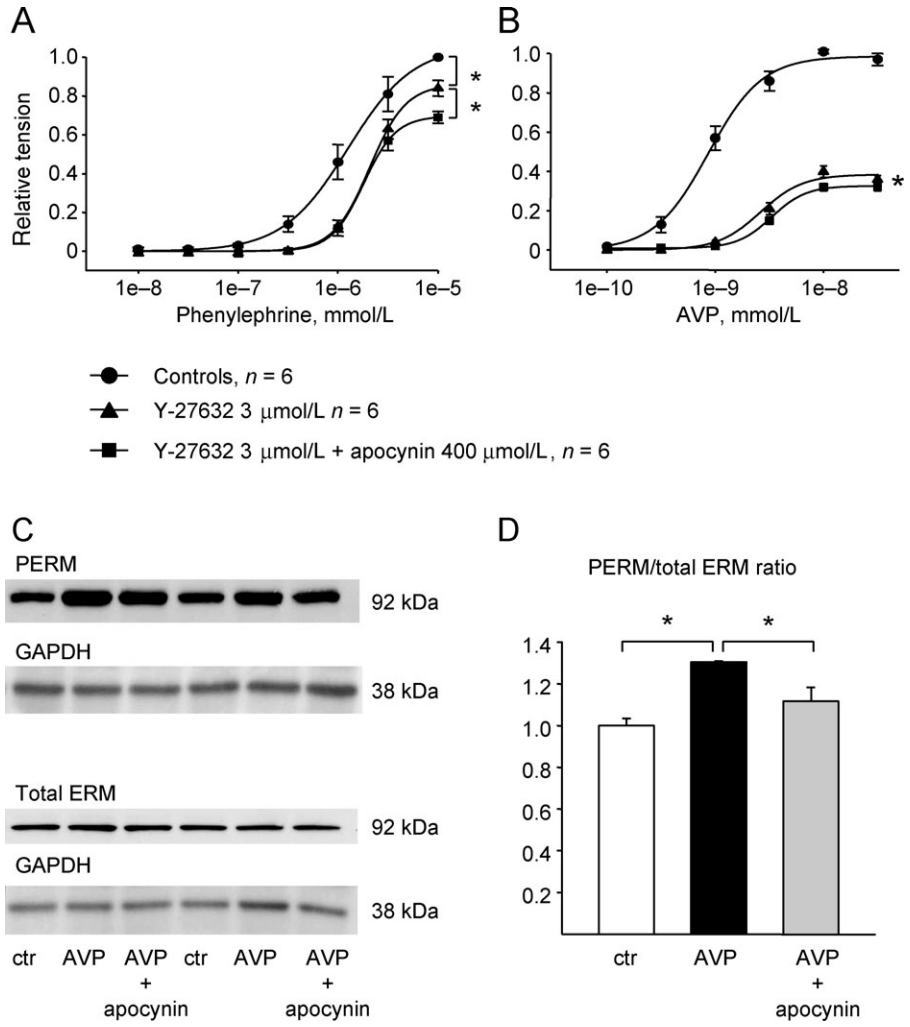

Figure 7 (*A*) Phenylephrine-induced constriction of renal interlobar arteries from F344 rats. Rho kinase inhibition significantly shifted logEC50 for phenylephrine to the right ($P < 0.05$) and reduced maximum tension (*$P < 0.05$). Combined pretreatment with the Rho kinase inhibitor and apocynin further reduced maximum tension (*$P < 0.05$). (*B*) Arg-vasopressin-induced constriction of renal interlobar arteries from F344 rats. Rho kinase inhibition significantly shifted logEC50 for arg-vasopressin to the right ($P < 0.001$) and reduced maximum tension ($P < 0.001$). Combined pretreatment with the Rho kinase inhibitor and apocynin did not further reduce maximum tension. (*C*) Western blots for ezrin, radixin, and moesin (ERM) as well as their Rho kinase-dependent phosphorylation products (PERM) in F344 rat aortic tissue. (*D*) PERM/total ERM ratios as a measure of Rho kinase activity. Data are normalized to the control group. Arg-vasopressin-treated aortas ($n = 3$) showed a significantly higher PERM/total ERM ratio than controls ($n = 3$) and aortas treated with apocynin and arg-vasopressin ($n = 4$) (*$P < 0.05$).

activate apocynin to inhibit NADPH oxidase activity in aortic tissue.

In contrast to the *in vitro* situation, pharmacokinetic studies in rats failed to provide evidence for the activation of apocynin after oral administration[13] or intraperitoneal injection.[31] Furthermore, apocynin concentrations used for *in vitro* investigations usually are in the high micromolar[14,24,30] or in the millimolar range.[12] Apocynin doses commonly used in experimental studies[24–27,31] *in vivo* lead to apocynin concentrations in the biophase that are approximately two to three orders of magnitude lower[31] than those frequently used *in vitro*. These data suggest that apocynin is a potent NADPH oxidase inhibitor when activated by peroxidase but that this activation usually does not occur in vascular tissue *in vivo*.

Since apocynin failed to inhibit vascular NADPH oxidase activity *in vivo*, we performed additional experiments to identify the mechanism(s) underlying the acute vascular effects of the drug. Our data show that Rho kinase inhibition with Y-27632 attenuated AVP-induced vasoconstriction in renal intralobar arteries.[32] Apocynin did not have an additive effect, suggesting that it may also act through Rho kinase inhibition. In fact, our measurements of Rho kinase activity in aortic tissue revealed that the enzyme was activated by AVP and that apocynin blunted the AVP-induced Rho kinase activation. These data suggest that the acute vasodilatory effects of apocynin may at least in part be due to Rho kinase inhibition.

Taken together, data from the present study and from others[13,30,31] suggest that rapid vasodilator actions of apocynin may not be critical for its potential antihypertensive actions because the desired apocynin concentrations in the biophase may not be reached with currently used dosing protocols. Data on vasomotor function obtained from experiments using high local *in vivo* or high *in vitro* apocynin concentrations should be re-interpreted as far as the NADPH oxidase is concerned because a large part of its vasodilator action cannot be attributed to interference with p47phox assembly with other NADPH oxidase subunits.

5. Conclusion

The identification of NADPH-oxidase-derived superoxide as an important pathogenic factor in cardiovascular disease including arterial hypertension has led to the search for substances that inhibit the NADPH oxidase activity for experimental and therapeutic aims. Development of non-peptide NADPH oxidase inhibitors is a complex task because NADPH oxidases have multimeric structures and exist as several isoenzymes with tissue-specific expression patterns and activation mechanisms. This issue is further complicated by the ubiquity of its electron-donating substrate NAD(P)H that participates in virtually every biochemical process and therefore may preclude the development of inhibitors that compete with this substrate. To enhance scientific progress in this particular field of hypertension research more rigorous testing of new NADPH oxidase inhibitors for isoenzyme specificity and potential receptors other than NADPH oxidases should be performed in relevant reduced experimental systems. This would facilitate the application of NADPH oxidase inhibitors in complex systems including *in vivo* administration and would allow hypothesis testing based on pharmacologic interventions and read out parameters different form NADPH oxidase activity.

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