Phosphoinositide 3-Kinase Mediates Enhanced Spontaneous and Agonist-Induced Contraction in Aorta of Deoxycorticosterone Acetate–Salt Hypertensive Rats

Carrie A. Northcott, Matthew N. Poy, Sonia M. Najjar, Stephanie W. Watts

Abstract—Arteries from deoxycorticosterone acetate (DOCA)-salt and N\textsuperscript{o}-nitro-L-arginine (L-NNA) hypertensive but not normotensive rats develop spontaneous tone. LY294002 and wortmannin, phosphoinositide 3-kinase (PI3-kinase) inhibitors, eliminate spontaneous tone. We hypothesized that PI3-kinase protein and/or activity was increased in hypertension and contributed to the observed enhanced contractility. PI3-kinase activity assays revealed 2-fold higher activity in thoracic aorta from DOCA-salt [systolic blood pressure (SBP) = 184 ± 5 mm Hg] compared with sham rats (SBP = 111 ± 2 mm Hg). Western analyses of aortic homogenates revealed the presence of p85\alpha, p110\alpha, p110\beta, and p110\delta but not p110\gamma PI3-kinase subunits; p110\delta protein was elevated in aorta of hypertensive rats as compared with sham. Aortic homogenates from L-NNA rats also had elevated p110\beta protein density, but neither L-NNA nor DOCA-salt had differences in p85\alpha and p110\alpha. Total Akt density was unaltered, but pAkt was significantly lower in homogenates from DOCA-salt rats. LY294002 (20 \textmu mol/L) and nifedipine (50 nmol/L) abolished Ca\textsuperscript{2+}-induced spontaneous tone in aorta from DOCA-salt rats. However, LY294002 did not alter BayK8644-induced contraction, indicating that LY294002 does not inhibit L-type Ca\textsuperscript{2+} channels directly. PTEN (phosphatase and tensin homolog) and pPTEN were expressed but not different in aorta from DOCA-salt and sham rats. LY294002 corrected the enhanced contraction to KCl and norepinephrine in aorta from DOCA-salt rats. These data support an increase in PI3-kinase activity and p110\delta density in aorta from L-NNA and DOCA-salt rats. Importantly, this increase contributes to the enhanced contractility observed in two models of hypertension. (Circ Res. 2002;91:360-369.)

Key Words: phosphoinositide 3-kinase • artery • deoxycorticosterone acetate–salt hypertension • phosphatase and tensin homolog • N\textsuperscript{o}-nitro-L-arginine hypertension

Phosphoinositide 3-kinase (PI3-kinase) is a multifaceted enzyme, possessing both lipid and protein kinase activity. It participates in multiple signaling pathways, receiving input from a variety of receptors ultimately leading to changes in gene expression, metabolism, cell division, apoptosis, cellular trafficking, and contractility.1–6 In general, PI3-kinase transfers a phosphate group to the D3 position of phospholipids. Cloning of the catalytic subunits has led to organizing the multigene family into 3 groups based on substrate specificity.1–6 Importantly, PI3-kinase has been implicated in the modulation of vascular and nonvascular smooth muscle contractility.7–11

Hypertension is a disease characterized by enhanced agonist-stimulated contraction, reduced agonist-stimulated relaxation, smooth muscle cell growth, and spontaneous arterial tone. Because PI3-kinase plays a role in modulating contraction and growth, it is logical to question the involvement of PI3-kinase in vascular changes associated with hypertension. Of specific interest to this work is spontaneous tone, where arteries contract with no exogenous stimulus and which can be associated with oscillatory contractions. Spontaneous tone occurs in arteries of hypertensive rats and is due, at least in part, to altered Ca\textsuperscript{2+} sensitivity and/or handling.12–16 All the Class I PI3-kinase subunits associate with L-type Ca\textsuperscript{2+} channels and increase current directly17 or via protein kinase C (PKC).18 The end result is an increase in intracellular Ca\textsuperscript{2+}. Thus, we investigated the profile of PI3-kinase proteins present in arteries and whether this profile is altered in arteries from 2 models of hypertension. We also assessed PI3-kinase Ca\textsuperscript{2+}-dependence with respect to mediating spontaneous tone in the aorta.

A potential mechanism of altered PI3-kinase activity is a change in phosphatase and tensin homolog (PTEN) activity. PTEN and several phosphatases are regulators of PI3-kinase activity. PTEN is a unique tumor suppressor gene that encodes a dual-specificity phosphatase and functions by removing the 3-phosphate from inositol moieties and proteins phosphorylated by PI3-kinase.19–22 PTEN exists primarily in the inactive phosphorylated state (pPTEN) and the phosphor-
ylation suppresses PTENs activity by preventing its recruitment to the PTEN-associated complex (PAC).

Thus, we determined if PTEN and pPTEN were present and whether they were altered between deoxycorticosterone acetate (DOCA)-salt and sham rat aorta.

Arterial responsiveness to NE is increased in hypertension and NE, as well as a host of other vasoactive compounds, and activates PI3-kinase in vascular smooth muscle and small arteries in the rat. Thus, enhanced arterial contraction to NE may be PI3-kinase mediated, and we have examined this idea. Overall, we tested the hypothesis that there is an increase in PI3-kinase protein and/or activity in hypertension that is ultimately responsible for physiologically relevant changes in contractility.

Materials and Methods

Surgical and Blood Pressure Protocol

DOCA-Salt Hypertension

Male Sprague-Dawley rats (250 to 300 g; Charles River Laboratories, Inc, Portage, Mich) underwent uninephrectomy and implantation...
Figure 2. A, Western blot of pAkt, reflective of PI3-kinase activity, of rat aortic smooth muscle cells exposed to vehicle, LY294002 (20 μmol/L), and LY303511 (20 μmol/L) with or without EGF (10 nmol/L). B, Representative tracing of spontaneous arterial tone in endothelium-denuded aorta from DOCA-salt hypertensive and sham normotensive rats. Tissues were under a constant tension for optimal force production; vehicle (0.1% DMSO) or EGF (10 nmol/L). B, Representative tracing of spontaneous tone. For Ca²⁺ experiments, arteries were first incubated in Ca²⁺-free -adrenergic agonist, phenylephrine (PE) (10 µmol/L; LY303511, 20 µmol/L) were added and allowed to equilibrate for 1 hour. Tension developed in aorta isolated from DOCA-salt (Figure 1A, panel C). pAkt density quantitation was performed using NIH Image (version 1.61).

Western Protocol

Protein Isolation

Aorta were cleaned, pulverized in liquid nitrogen and solubilized in lysis buffer (0.5 mol/L Tris HCl [pH 8.6], 10% SDS, 10% glycerol) with protease inhibitors (0.5 mmol/L PMSF, 10 µg/mL aprotinin and 10 µg/mL leupeptin). Homogenates were centrifuged (11 000 g for 10 minutes, 4°C), and supernatant total protein measured. Vascular smooth muscle cells were derived from aorta of male Sprague-Dawley rats in an explant method described previously.33

Western Blotting

Equivalent amounts of aortic protein from sham, DOCA-salt, and L-NNA rats were separated on 7% SDS-polyacrylamide gels and transferred to Immobilon-P membrane for standard Western analyses using p85α (1:100; Upstate Biotechnology), p110α (1:250; BD Transduction Laboratories), p110β, p110δ, p110γ (1:1000; Santa Cruz Biotechnology, Inc), PTEN, pPTEN, Akt, and pAkt (1:100; Cell Signaling) antibodies. Positive controls for p110α were Jurkat cells (BD Transduction Laboratories); p110γ were K-562 cells; p110δ were U-87 MG cells; p110γ were U-937 cells; PTEN were PTEN(FL) epitopes (Santa Cruz Biotechnology, Inc); and pPTEN positive control were EGF-stimulated A431 cells (Upstate Biotechnology). Smooth muscle α-actin (1:400; Oncogene) was used as a marker to ensure equal loading of protein.

Immunoprecipitation and PI3-Kinase Activity Assay

Aorta were cleaned, pulverized in liquid nitrogen, and solubilized in PI3-kinase lysis buffer. p85α antibody (5 µL) and protein A agarose beads (70 µg/mL leupeptin) were added to equal amounts of total protein and the samples rocked (4°C) for 2 hours. The PI3-kinase assay was performed as previously described.33,34 Briefly, the p85α aortic homogenate immunoprecipitants from DOCA-salt and sham rats were incubated with phosphatidylinositol (PI) in the presence of [32P]adenosine triphosphate (ATP). Reactions were terminated with 15 µL 4N HCL and phospholipids extracted with 130 L CHCl₃ / methanol (1:1). The radioactive product of the reaction (PI3-monophosphate) was detected using thin layer chromatography (TLC) and quantified with Bio-Rad software.

Materials

Materials included acetylcholine hydrochloride, DOCA, BayK8644, L-NNA, nifedipine, NE, PE, KCl, wortmannin (Sigma Chemical Co), EGF (Gibco Life Technologies), LY294002 (Biomol), and LY303511 (gift from Dr Chris Vlahos, Eli Lilly, Indianapolis, Ind).

Data Analyses

Data are presented as mean±SEM for the number of animals (n) stated. Contraction is reported as tension (milligrams) or as a percentage of response to maximum contraction to PE, NE, or KCl. EC₅₀ values were determined using GraphPad Prism and reported as the mean of the negative logarithm (~log) of the EC₅₀ value. Band density quantitation was performed using NIH Image (version 1.61). When comparing 2 groups, the appropriate Student’s t test was used. An ANOVA followed by Student-Newman-Keuls post hoc tests were performed when comparing 3 or more groups. A value of P≤0.05 was considered statistically significant.

Results

Spontaneous Tone and PI3-Kinase

The systolic blood pressures of DOCA-salt and sham rats were 184±5 and 111±2 mm Hg, respectively. Spontaneous tone developed in aorta isolated from DOCA-salt (Figure 1A,
2nd tracing) but not sham rats (Figure 1A, 1st tracing). LY294002 (20 μmol/L), a specific and reversible PI3-kinase inhibitor, significantly reduced spontaneous tone (Figures 1A and 1B). Similarly, LY294002 (20 mol/L) inhibited spontaneous tone in aortic strips with the endothelium-intact from DOCA-salt rats (vehicle, 11001.6 11006 1.5% versus LY294002, 1100213.9 11006 5.6% initial PE (10 5mol/L) contraction). Systolic blood pressures of the L-NNA and sham rats were 200 121 mm Hg, respectively. Similar to the DOCA-salt model, spontaneous arterial tone developed (Figures 1C, 4th tracing) in aortic strips from the L-NNA model and not in sham. LY294002 (20 μmol/L) reduced the spontaneous tone that developed (Figures 1C and 1D). Increasing concentrations of LY294002 (10 7 to 3×10 4 mol/L) were added to tissues from DOCA-salt rats in the absence of agonist and spontaneous tone monitored to ensure that the concentration utilized in the experiments (20 μmol/L) was appropriate.

LY294002 reduced spontaneous tone in a concentration-dependent manner (Figures 1F and 1H) in the aortic strips from the DOCA-salt rats.

To ensure that LY294002 was acting selectively, we utilized LY303511, an inactive analog of LY294002. In contrast to LY294002, LY303511 did not inhibit epidermal growth factor (EGF)-induced activation of pAkt, a substrate of PI3-kinase, in cultured aortic smooth muscle cells (Figure 2A); Akt protein density was similar in all samples. LY303511 (20 μmol/L) failed to inhibit spontaneous tone (Figure 2B), but elicited a contraction in the aorta from DOCA-salt rats without altering tone of sham aorta.

PI3-Kinase Biochemistry

Having established a functional change in PI3-kinase in aorta from DOCA-salt rats, we proceeded to measure PI3-kinase activity. As Figure 3A reveals, p85α-associated PI3-kinase activity was significantly greater in aorta from DOCA-salt rats as compared with sham (5.7±0.9 versus 2.8±0.9 adjusted volume optical density×mm², respectively). Equal amounts of p85α protein were present in the immunoprecipitates from DOCA-salt and sham rats (Figure 3B), and this was confirmed with standard Western analyses (Figure 3C).
To examine how this alteration in activity may affect proteins downstream of PI3-kinase, we measured Akt and pAkt protein in aortic lysates from DOCA-salt and sham rats. Similar concentrations of Akt were found, but there was significantly lower pAkt protein density in aortic lysate from DOCA-salt rats as compared with sham (Figure 3D). These data suggested the enhanced PI3-kinase activity was being funneled to an alternative effector.

A profile of p110 subunits was next performed using Western analyses. The Class IA catalytic subunits p110α, p110β, and p110δ, but not p110γ, were present in the aorta of both DOCA-salt and sham rats, with p110δ being significantly higher in the aorta from DOCA-salt as compared with sham rats (P < 0.05) (Figure 4). Confirmation of the specificity of the antibodies was determined by examining a positive control for each antibody. These data suggest that the increase in p85α-associated PI3-kinase activity may be due to the increase in the p110δ subunit, because it is the only PI3-kinase subunit that exhibited greater density in the aorta from DOCA-salt as compared with sham rats.

We also examined aortic lysates from hypertensive L-NNA and normotensive sham rats. p85α, p110α, p110β, and p110δ, but not p110γ PI3-kinase subunits, were detected (Figure 5). There were significantly higher p110β and p110δ protein densities in the L-NNA compared with sham (Figures 5C). There were also no significant changes in pAkt (Figure 5F). These data demonstrate that both models have alterations in arterial PI3-kinase, suggesting this is common to hypertension.

Ca2+, PI3-Kinase, and Spontaneous Tone
The role of Ca2+ in mediating arterial spontaneous tone is established. Nifedipine, an L-type Ca2+ blocker, inhibited spontaneous tone in a manner similar to LY294002 (compare Figures 1A, 1C, and 6A). This concentration of nifedipine was the minimum concentration that maximally inhibited KCl-induced contraction in aorta. To ensure that LY294002 did not directly inhibit L-type Ca2+ channels, we examined the effect of LY294002 (20 μmol/L) on BayK8644, a direct L-type channel agonist. In agreement with others, we observed an enhanced contraction to BayK8644 in aorta from the DOCA-salt rat. LY294002 did not alter BayK8644-induced contraction (Figure 6B), indicating that LY294002 was not acting directly to inhibit L-type Ca2+ channels. To link Ca2+ and PI3-kinase, all Ca2+ was removed from the tissue bath and added back in increasing concentrations in the presence of LY294002 or vehicle and spontaneous tone development measured. LY294002 completely inhibited the development of spontaneous tone in aorta from DOCA-salt rats, whereas vehicle-incubated aorta developed calcium-dependent spontaneous tone (Figure 6C). Aorta from sham rats did not develop spontaneous tone. Additionally, KCl-induced arterial contraction depends on Ca2+ channel activation but may necessitate the activation of other signaling pathways. LY294002 did not shift KCl-induced contraction in arteries from sham rats. However, LY294002 significantly shifted KCl-induced contraction in arteries from DOCA-salt rats, back to that similar in sham (Figure 6D).

PTEN
We next examined aortic homogenates for the presence of PTEN and its inactive form, pPTEN. We found both PTEN and pPTEN in the thoracic aorta of both DOCA-salt and sham rats (Figure 7), but density of these proteins was not different between the aorta of DOCA-salt and sham rats. Appropriate positive controls were used to confirm antibody specificity. To our knowledge, this is the first time that PTEN and pPTEN have been localized to the arterial tissue.

Norepinephrine and PI3-Kinase
We last examined the effect of LY294002 on NE-induced contraction, a contraction that is enhanced in DOCA-salt
hypertension (Figure 8). LY294002 shifted the NE-induced aortic contraction of sham and the DOCA-salt rats compared with vehicle treated control tissues in both endothelium-denuded (−E) (representative tracing, Figure 8A) and endothelium-intact (+E) tissues, supporting a role for PI3-kinase in NE-induced contraction. Interestingly, the potency of NE in the presence of LY294002, in aorta from DOCA-salt and sham rats incubated with LY294002, was not significantly different (−log EC50 [M] sham−E=7.2±0.1; DOCA-salt−E=7.2±0.1; sham+E=6.6±0.1; DOCA-salt+E=6.4±0.1) (Figures 8B and 8C). Thus, an alteration in PI3-kinase may partially explain the enhanced arterial contractility to NE observed in DOCA-salt hypertension.

Discussion
Vascular growth and hyperresponsiveness in hypertension has been observed for decades. PI3-kinase plays a key role in cellular growth and apoptosis and has been implicated in modulating vascular contraction. LY294002 given acutely in vivo to conscious hypertensive DOCA-salt rats lowered blood pressure (data not shown), but also lowered heart rate, making it difficult to determine the role PI3-kinase in hypertension in the vasculature of conscious animals. With this in mind, we examined alterations in PI3-kinase activity and protein in DOCA-salt and L-NNA hypertension and how this alteration affects vascular contraction in aorta isolated from these models.
Our main hypothesis stemmed from the initial finding that LY294002 eliminated spontaneous tone in aorta from a DOCA-salt hypertensive and sham normotensive rats. Tissues were under a tension for optimal spontaneous tone in aorta from DOCA-salt and sham rats. The reason for this contraction was unclear, but LY303511 did not inhibit PI3-kinase activity as it did not reduce EGF-induced phosphorylation of Akt. Interestingly, the contraction occurred only in the aorta from DOCA-salt and not sham rats.

**PI3-Kinase Biochemistry**

To assay for PI3-kinase activity, we used an antibody against p85α. We chose to use this antibody versus a phosphotyrosine antibody because of the specificity for PI3-kinase association provided by p85α. There were no significant differences in the p85α protein levels, but there was significantly elevated PI3-kinase activity in aorta from DOCA-salt rats as compared with sham.

This led us to inquire if the increase in activity was associated with an increase in p110 protein density. We detected p110α, p110β, and p110δ in the thoracic aorta of DOCA-salt, L-NNA, and sham rats with a significantly greater amount of p110δ in the aorta of DOCA-salt and L-NNA compared with the sham rats, as well as a significantly greater p110β in the L-NNA model of hypertension. This is in contrast to the findings of Macrez et al. in which p110α, p110β, and p110γ were found, but p110δ, in rat portal vein myocytes. The localization of p110δ to the aorta was unexpected because p110δ had been reported to be restricted to hematopoietic cells. The upregulation of p110 subunit density may constitute a potential mechanism of the enhanced PI3-kinase activity observed. It is unlikely changes in PTEN function contribute to this increase in activity as PTEN density and state of activation was not different in DOCA-salt and sham tissue. This is reflected in the L-NNA model of hypertension.

Our findings of dramatically enhanced contraction to BayK8644 in aorta from DOCA-salt rats supported previous observations in coarctation-hypertensive rats, DOCA-salt hypertensive rats, L-NNA, and spontaneously hypertensive rats (SHR) compared with their respective normotensive controls. These data suggest an upregulation of L-type

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*Figure 6.* A, Representative tracing of spontaneous arterial tone in endothelium-denuded aorta from DOCA-salt hypertensive and sham normotensive rats. Tissues were under a tension for optimal spontaneous tone in aorta from DOCA-salt and sham rats. LY294002 or vehicle was added to the tissues 1 hour before cumulative addition of BayK8644. B, Effect of L-type voltage gated Ca²⁺ channel agonist BayK8644 and PI3-kinase antagonist LY294002 (20 μmol/L) in endothelium-denuded rat aorta from DOCA-salt and sham rats. LY294002 or vehicle was added to the tissues 1 hour before cumulative addition of BayK8644. C, Effect of Ca²⁺ chloride and PI3-kinase antagonist LY294002 in endothelium-denuded rat aorta from DOCA-salt and sham rats. LY294002 or vehicle was added to the tissues 1 hour before cumulative addition of Ca²⁺ chloride. Data are presented as a percentage of the initial phenylephrine (PE) (10⁻⁵ mol/L) contraction. D, Effect of KCl and PI3-kinase antagonist LY294002 (20 μmol/L) in endothelium-denuded rat aorta from DOCA-salt and sham rats. LY294002 or vehicle was added to the tissues 1 hour before cumulative addition of KCl. Points represent mean±SEM. *P<0.05.
calcium channel activity in DOCA-salt rat hypertension. Recently, Molero et al.\(^44\) found an increase in L-type Ca\(^{2+}\) channel expression in membrane protein from DOCA-salt compared with sham rats in small mesenteric arteries. Viard et al.\(^18\) demonstrated that G\(\beta\gamma\) dimers have the ability to stimulate vascular L-type Ca\(^{2+}\) channels through PI3-kinase, which adds support to the notion that PI3-kinase and L-type Ca\(^{2+}\) channels are linked and G protein-coupled receptors have the potential to tap into this interaction. Macrez et al.\(^17\) demonstrated that p110 subunits can directly associate with L-type Ca\(^{2+}\) channels. Thus, if there is an alteration in PI3-kinase activity, this may further activate Ca\(^{2+}\) channels. The regulation of Ca\(^{2+}\) flux by PI3-kinase was strengthened by the finding that LY294002 inhibited all Ca\(^{2+}\)-induced spontaneous tone as well as corrected the enhanced KCl-induced contraction in aorta from DOCA-salt rats. We realize that KCl and NE are mechanistically different in how they induce vascular contraction, but LY294002 corrected the enhanced contraction observed in aorta from DOCA-salt rats in both types of contraction. Thus, this supports a role for PI3-kinase in some aspect of contraction for both compounds. At the present time, it is unclear whether a change in Ca\(^{2+}\) channels, Ca\(^{2+}\) flux, PI3-kinase, or a combination of these is sufficient to enable the development of arterial spontaneous tone and hyperresponsiveness observed in hypertension.

However, PI3-kinase may be connected to Ca\(^{2+}\) channels, their interaction has implications for the hyperresponsiveness to contractile agonists that is characteristic in hypertension. LY294002 normalized NE-induced contraction in sham and DOCA-salt tissues, indicating that it is the increased activity of PI3-kinase that is responsible for enhanced contraction to NE. Because PI3-kinase is integral to signaling of hormones involved in contraction and growth, an increase in PI3-kinase activity has significant implications for arterial function.

**Limitations**

Several limitations of these studies should be acknowledged. In the studies we performed, we used only the thoracic aorta. We chose to do so because the aorta is well characterized and easier to use for protein isolation. We are cognizant of the fact that this is not a resistance artery, and thus, we cannot state that our observations apply to resistance arteries. We also recognize that there are limitations with the PI3-kinase inhibitors. LY294002 has the ability to inhibit casein kinase 2 (CK2).\(^45\) Because of the lack of selective CK2 inhibitors, it was not possible to control for this limitation. Wortmannin, another PI3-kinase inhibitor, inhibits myosin light chain kinase.\(^45\) We chose to focus our studies using the inhibitor LY294002 after finding that both inhibitors inhibited spontaneous tone, as well as the fact that we had a control for LY294002. LY303511. Finally, we have studied alterations in PI3-kinase only after the development of hypertension.
making it difficult to draw any conclusions when these changes in the vasculature occur.

Summary
In conclusion, we have demonstrated a role for PI3-kinase in spontaneous tone development and hypertreactivity in thoracic aorta from DOCA-salt and L-NNA rats. This may, in part, be due to an increase in PI3-kinase activity via increased p110δ and p110β protein density observed in aorta of hypertensive rats. We are the first, to our knowledge, to perform a complete profile of Class I p110 PI3-kinase subunits in the aorta. Alteration in activity/protein may be linked to calcium modulation, perhaps one mechanism is via L-type calcium channels. This is also the first demonstration of the presence of PTEN and pPTEN in arterial tissue. Increased PI3-kinase activity has functional significance in that when PI3-kinase is inhibited spontaneous tone, KCl and NE-induced hypertreactivity is decreased. Collectively, these data provide an argument for the importance of PI3-kinase in the vascular smooth muscle and in hypertension.

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