PI3-Kinase Upregulation and Involvement in Spontaneous Tone in Arteries From DOCA-Salt Rats

Is p110δ the Culprit?

Carrie A. Northcott, Joel S. Hayflick, Stephanie W. Watts

Abstract—Increased expression of phosphoinositide 3-kinase (PI3-kinase) mediates elevated tone in the aorta from hypertensive deoxycorticosterone acetate (DOCA)-salt rats. In this article, we hypothesized that (1) alterations observed with respect to PI3-kinase observed in the aorta would also occur in mesenteric resistance arteries responsible for determining total peripheral resistance (TPR) and (2) p110δ activity was increased and localized to vascular smooth muscle cells (VSMCs), and was responsible for the increase in spontaneous tone in aortae from DOCA-salt rats. Mesenteric resistance arteries and aorta were isolated from DOCA-salt (190±3 mm Hg) and sham (121±2 mm Hg) rats. Myograph experiments revealed LY294002 (20 μmol/L), a PI3-kinase inhibitor, significantly decreased tone in mesenteric resistance arteries from DOCA-salt rats as compared with sham (−49±12 mg versus −10±7 mg). Western analyses of resistance artery protein homogenate revealed p85α and p110δ subunit protein, with significantly elevated levels of p110δ protein in the DOCA-salt compared with sham rats (0.30±0.07 versus 0.16±0.04% smooth muscle alpha-actin arbitrary units). Immunohistochemistry revealed p110δ-specific staining in VSMCs, with more intense staining in aortae from DOCA-salt rats. Compared with aortae from sham, p110δ-associated PI3-kinase activity was increased in DOCA-salt (158% of sham) and likely responsible for spontaneous tone because the p110δ specific inhibitor IC87114 decreased spontaneous tone in a concentration-dependent manner. Collectively, these data further implicate the p110δ isoform of PI3-kinase in arterial hyperresponsiveness in hypertension at the level of both large and small arteries. (Hypertension. 2004;43:1-6.)

Key Words: arteries • hypertension, mineralocorticoid

PI3-kinase is a signaling enzyme that plays key roles in cellular growth, remodeling, and apoptosis and is implicated in modulating vascular contraction.1–9 PI3-kinase possesses both lipid and protein kinase activity, giving it the ability to be involved with a great number of signaling pathways. Cloning of the catalytic subunits of PI3-kinase led to organizing the multigene family into 3 main classes based on their substrate specificity, sequence homology, and regulation. Class I PI3-kinases are the most extensively investigated class and contain two subunits, one of which plays primarily a regulatory/adaptor role (p85α, β, p55γ and p101) and the other that maintains the catalytic role of the enzyme (p110α, β, δ, and γ).1–6

Spontaneous tone (non-agonist-induced contraction) is a phenomenon that is observed in both experimental and clinical forms of hypertension. Spontaneous tone has been observed in femoral arteries from renal hypertensive rats, deoxycorticosterone acetate (DOCA)-salt hypertensive rats, rat genetically predisposed to hypertension, essential hypertensive patients, and women with preeclampsia.7,10–12 Spontaneous tone development in the condition of hypertension leads to “spontaneous” narrowing of the arteries that can further increase/propagate the condition of hypertension by altering total peripheral resistance (TPR). Two structurally unrelated pharmacological inhibitors of PI3-kinase—LY294002 and wortmannin—inhibited aortic spontaneous tone observed in DOCA-salt rats in a concentration-dependent manner. Moreover, Class IA regulatory p85α subunit-associated PI3-kinase activity and PI3-kinase protein expression, specifically the p110δ subunit, was upregulated in aorta from DOCA-salt hypertensive rats compared with normotensive sham animals.7

It is not apparent how different p110 isoforms play specific functional roles in cells. Moreover, the aorta, a conduit artery, is the tissue in which most of the characterization of PI3-kinase and hypertension has been studied and only has a relatively small influence on blood pressure. Therefore, we hypothesized that alterations observed with respect to PI3-kinase observed in aorta would also be observed in mesenteric resistance arteries. Furthermore, we hypothesized p110δ
would be localized to vascular smooth muscle cells (VSMCs) in aorta, be responsible for the increase in PI3-kinase activity, and contribute specifically to the altered tone observed in aorta from DOCA-salt rats.

Methods

Surgical and Blood Pressure Protocol
Male Sprague Dawley rats (250 to 300 g; Charles River Laboratories, Inc., Portage, Mich) underwent uninephrectomy and implantation of DOCA (200 mg/kg) under isoflurane anesthesia. Animals remained on the regimen for 4 weeks. Systolic blood pressures were measured using standard tail cuff methods.

Isolated Tissue Bath Protocol
Endothelial cell-denuded thoracic aorta removed from pentobarbital (60 mg/kg, IP) anesthetized rats, were pair-mounted (Sham/DOCA) in isolated tissue baths for measurement of isometric force. Tissues were challenged with a maximal concentration of α adrenergic agonist, phenylephrine (PE) (10^{-5} mol/L). IC87114 concentration response curves were generated by adding increasing concentrations of IC87114 (1×10^{-9} to 3×10^{-4} mol/L) with measurements of spontaneous tone taken every 30 minutes. Aortic strips from DOCA-salt rats were also exposed to 20 μmol/L IC87114 or vehicle for 1 hour and measurements of spontaneous tone were recorded.

Myograph Protocol
Small mesenteric resistance arteries (2 to 3 mm long, 200 to 300 μm diameter) were dissected away from mesenteric veins under a light microscope and mounted between 2 tungsten wires in a dual-chamber wire myograph for measurements of isometric force. DOCA-salt rat (200 to 300 μm diameter) in endothelium-denuded mesenteric resistance arteries from a DOCA-salt rat was paired (Sham/DOCA) in isolated tissue baths for measurement of isometric force. Tissues 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 μL CHCl/methanol (1:1). The radioactive product of the reaction (PI3-monophosphate) was detected using thin layer chromatography (TLC) and quantified with Biorad and National Institutes of Health (NIH) image (v.1.61) software.

Data Analyses
Data are presented as means ±SE of the number of animals (n) stated. Contraction is reported as tension (milligrams) or as a percentage of response to maximum contraction to PE. Quantitation of band density from Western blot analyses were performed on computer-scanned images of developed films using NIH Image (v.1.61). PI(3)P radiolabeled areas were quantified using the program Bio-Rad Quantity One and NIH Imaging Software. When comparing 2 groups, the appropriate Student t test was used. An ANOVA followed by Student Newman Keuls post hoc tests were performed when comparing 3 or more groups. In all cases, a P<0.05 was considered statistically significant.

Materials
Materials included acetylcholine hydrochloride, DOCA, phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, Mo), LY294002 (Biomol, Plymouth Meeting, Pa), and IC87114 (ICOS Corporation, Bothell, Wash).

Figure 1. A, Representative tracing of spontaneous arterial tone in endothelium-denuded mesenteric resistance arteries from a DOCA-salt rat (200 to 300 μm diameter). Tissues are under passive tension for optimal force production; vehicle (0.1% DMSO) or LY294002 (20 μmol/L) was added and allowed to equilibrate for 1 hour. The arrow represents the baseline at which quantification of the LY294002-induced relaxation was compared. B, Effect of PI3-kinase inhibitor LY294002 or vehicle on spontaneous tone in endothelium-denuded rat aorta from DOCA-salt and sham rats. Bars represent the LY294002 or vehicle-induced relaxation (milligrams) in the mesenteric resistance arteries ±SEM. *Statistically significant difference (P<0.05) between DOCA-salt Vehicle and LY294002 treatment groups.

Materials
Materials included acetylcholine hydrochloride, DOCA, phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, Mo), LY294002 (Biomol, Plymouth Meeting, Pa), and IC87114 (ICOS Corporation, Bothell, Wash).
Results

The systolic blood pressure of the DOCA-salt and sham rats were 190±3 mm Hg and 121±2 mm Hg, respectively. Resistance arteries, approximately 240 μm in diameter, were placed in a myograph for measurements of isometric force. Elevated tone (excluding oscillations) developed in several (46%), but not all, of the resistance arteries removed from the DOCA-salt rats (Figure 1A). Spontaneous tone did not develop in resistance arteries removed from sham rats. LY294002 (20 μmol/L) significantly inhibited tone in the resistance arteries from DOCA-salt rats as compared with sham or vehicle-incubated arteries from DOCA-salt rats (Figure 1A and 1B).

To examine the resistance arteries biochemically, vessels (200 to 300 μm diameter) were removed from sham and DOCA-salt animals, pooled, and total protein was isolated. Western analyses revealed the presence of p85α, p110δ, Akt, and pAkt protein in resistance arteries from both sham and DOCA-salt rats (Figure 1A through 1B). Akt is a signaling enzyme phosphorylated by PI3-kinase and is commonly used to examine PI3-kinase activity in cells. There was significantly greater Class IA catalytic PI3-kinase subunit p110δ protein in resistance arteries from DOCA-salt rats compared with sham, however no differences were found between vessels from sham and DOCA-salt rats with respect to the p85α, Akt and pAkt protein (Figure 2A through 2C). p110δ and p85α were the only two PI3-kinase subunits examined due to the limited amount of protein and the fact that previous studies have demonstrated that p110δ was the subunit altered in aorta from DOCA-salt hypertensive rats.7

We next determined whether p110δ could be observed in VSMCs. Immunohistochemical studies revealed p110δ specific staining in the smooth muscle cell region in the aortae of both the sham and DOCA-salt rats (n=4) (Figure 3A, arrows). The aorta from the DOCA-salt rat had more intense staining than that of the sham, supporting the increase in p110δ protein observed in aorta from DOCA-salt rat.7 To further investigate the involvement of PI3-kinase p110δ subunits in enhanced aortic PI3-kinase activity, p110δ-specific PI3-kinase activity assays were performed. Figure 3B
illustrates a significant increase in p110δ-associated PI3-kinase activity in the aorta from the DOCA-salt rat compared with the sham (158% of sham). Immunoprecipitation with the p110δ antibody confirmed that the antibody reacted only to the p110δ subunit and no other p110 subunits (Figure 3C). This lends support to the hypothesis that increased p110δ PI3-kinase activity may mediate enhanced p110δ-mediated tone in aorta from DOCA-salt rats.

Spontaneous tone developed in aorta from DOCA-salt but not sham rats [Figure 4A (1st and 2nd tracing) and 4B]. Increasing concentrations of IC87114 (10^{-9} to 3\times10^{-9} \text{mol/L}) or vehicle (DMSO) were added to endothelium-denuded aortic strips from DOCA-salt rats in the absence of agonist, and spontaneous tone was monitored. IC87114 reduced spontaneous tone in a concentration-dependent manner (Figure 4A and 4B). The effect of IC87114 was reversible in all experiments, as spontaneous tone was restored on washing out of IC87114. Finally, equimolar concentrations of LY294002 (20 \text{µmol/L}), IC87114 (20 \text{µmol/L}), or vehicle (0.1% DMSO) were incubated with aortic strips from DOCA-salt rats for 1 hour in isolated tissue baths. These experiments further demonstrated that IC87114 significantly inhibits spontaneous tone development in DOCA-salt rats compared with vehicle (Figure 4C).

Discussion

Previous studies examining alterations in PI3-kinase–mediated spontaneous tone used the aorta as the vessel of choice. The aorta is a conduit artery and has recently been found to play at least a small role in the maintenance of blood pressure, due to changes in compliance in the aorta during the condition of hypertension. More immediately relevant to control of TPR is the function of resistance arteries. Resistance arteries from sham rats did not develop elevated tone, and the arteries from DOCA-salt rats displayed variable levels of elevated tone. However, small changes in the diameter of resistance arteries can lead to large changes of TPR due to their relationship (R_{1/r^4}). When LY294002 was added to resistance arteries from DOCA-salt rats, the tone of the arteries was significantly reduced. Because LY294002 had no effect on nor did spontaneous tone develop in resistance arteries and aorta from sham rats, changes in PI3-kinase activity were specific to the arteries from hypertensive animals. This was further evidenced by Western analyses in...
which, similar to the aorta, a significant increase in the p110δ subunit was observed in resistance arteries from DOCA-salt hypertensive rats. Moreover, there was no increase in p85α, Akt, and pAkt in mesenteric arteries from DOCA-salt rats compared with sham; this observation was also made in aortae. These studies further suggest that phosphorylation of Akt may not be an absolute measure of changes in PI3-kinase activity because PI3-kinase may have targets independent of Akt. Collectively, these results further demonstrate that PI3-kinase is a key component in spontaneous tone development in small as well as large arteries from DOCA-salt rats, suggesting PI3-kinase plays a crucial role in hypertension-related elevated tone.

The catalytic PI3-kinase subunit p110δ is involved in multiple pathways, including specific signaling pathways of neutrophil activation, antigen receptor signaling in T and B lymphocytes, and insulin signaling. p110δ, until recently, has been thought to reside primarily in hematopoietic cells. Therefore, it was a surprise when p110δ protein was found in aortae of hypertensive DOCA-salt, LNNA, and normotensive sham rats. Moreover, there was significantly greater p110δ protein in the aortae of DOCA-salt and LNNA rats compared with their normotensive controls, thus implicating PI3-kinase p110δ subunit involvement in hypertension. These data led to the hypothesis that the increase in PI3-kinase activity and spontaneous tone observed in DOCA-salt hypertension was associated with alterations in the p110δ subunit. Immunohistochemical studies confirmed that the p110δ PI3-kinase subunit was indeed in the VSMC. More intense staining was also observed in the VSMC in the aortae from DOCA-salt rats compared with sham, thus further supporting the increase in p110δ protein in aortae from DOCA-salt rats. Our findings of nonhematopoietic localization of p110δ was recently supported in studies demonstrating p110δ mRNA in nonhematopoietic tissues such as lung, heart, placenta, and brain. Furthermore, Sawyer et al retrieved expressed sequence tag (EST) data from the GenBank database to examine tissue distribution of the p110 catalytic subunits. These studies revealed that the p110δ subunit ESTs were distributed with 39% being in blood/arteries, 34% in the heart, and 17% in the brain. Additionally, p110δ ESTs were detected in aortic endocardium, whereas p110α ESTs were exclusively detected in hematopoietic cells.

**Figure 4.** A, Representative tracings of vehicle and IC87114 (10^{-9} to 3\times10^{-5} mol/L) concentration response curves in endothelium-denuded aorta from DOCA-salt and sham rats. Tissues were under passive tension for optimal force production. B, The effect of increasing concentrations of IC87114 or vehicle on spontaneous tone in aorta from DOCA-salt and sham rats. Points represent means ± SEM. C, The effect of IC87114 (20 µmol/L), LY294002 (20 µmol/L), or vehicle (0.1% DMSO) (incubated for 1 hour) on spontaneous tone in aorta from DOCA-salt and sham rats. Data are presented as a percentage of the initial phenylephrine (PE) (10^{-5} mol/L) contraction. Bars represent means ± SEM. *Statistically significant difference (P<0.05) between DOCA-salt vehicle and treatment groups.

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immune cells, as expected, but 18% of p110δ ESTs fell into the "other" category representing 18 different tissues. These findings further support the idea that p110δ is not restricted to hematopoietic cells. We realize an alternative conclusion are that the results of our studies and those by Sawyer et al23 were altered due to the presence of leukocytes remaining in the tissues at the time of harvest. While we have not ruled this out, this is unlikely.

The upregulation of p110α subunit density may constitute a potential mechanism for the enhanced PI3-kinase activity observed in aortae. PI3-kinase activity assays in which the p110δ antibody was used demonstrated an increase in p110δ-mediated PI3-kinase activity. This was further examined by using a newly available specific inhibitor of p110, IC87114. The IC50 values for IC87114 are 0.5 μmol/L for p110δ, >29 μmol/L for p110α, >75 μmol/L for p110β, and >100 μmol/L for p110ε.18 IC87114 inhibited spontaneous tone in the aorta from DOCA-salt rats, at concentrations that do not significantly affect the other p110 subunits present in the aorta. p110γ, the PI3-kinase subunit most likely to be affected by a higher concentration of IC87114, has not been found in the aorta.2 Thus, these data demonstrate an increase in p110δ-associated arterial PI3-kinase activity and p110δ protein in the condition of hypertension and that this upregulation may explain the PI3-kinase-mediated spontaneous tone that develops.

Perspectives

PI3-kinase has been implicated in a broad spectrum of physiological processes as well as in the pathology of diseases such as cancer, metabolic, inflammatory, and cardiovascular diseases. However, the lack of specific tools has limited the ability to elucidate which subunits are responsible for the PI3-kinase alterations observed. Recently, with development of better antibodies, p110γ and p110δ PI3-kinase null-mice, and several subunit-specific inhibitors, the complexity of PI3-kinase is beginning to be unraveled. Results from studies with PI3-kinase p110δ and p110δ-null mice have suggested that these subunits are promising drug targets for allergies, chronic inflammation, cardiovascular areas, and autoimmune diseases. The data presented here add further promise to the use of p110δ as a potential drug target.

To summarize, these data support an increase in PI3-kinase-mediated spontaneous tone and an increase in PI3-kinase protein, specifically the p110δ subunit in the mesenteric resistance arteries. These data emphasize the importance of the p110δ PI3-kinase subunit via localization to VSMCs, upregulation of p110δ-specific PI3-kinase activity and expression, and pharmacological evidence that the p110δ subunit is a mediator of spontaneous tone. Further experiments with other p110-specific inhibitors and/or genetically altered animals will have to be performed to determine if p110δ is truly responsible for the enhanced contraction and spontaneous tone observed in hypertension and contributions made to this disease.

References

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