

Angiotensin II Signaling to Phospholipase D in Renal Microvascular Smooth Muscle Cells in SHR

Bradley T. Andresen, Edwin K. Jackson, Guillermo G. Romero

Abstract—Angiotensin II (Ang II)–induced phospholipase D (PLD) activity is greater in aortic smooth muscle from spontaneously hypertensive rats (SHR) versus normotensive Wistar-Kyoto rats (WKY). Whether and how this signaling pathway is altered in preglomerular microvascular smooth muscle cells (PGSMCs), a cell type that may participate in genetic hypertension, is unknown. The goals of the present study were to determine in SHR and WKY PGSMCs the following: (1) whether Ang II induces PLD activity; (2) whether the effect of Ang II on PLD activity is greater in SHR; (3) which PLD isoform is stimulated by Ang II; (4) what signaling pathway mediates Ang II–induced PLD stimulation; and (5) whether the signaling pathways mediating Ang II–induced PLD activity are different in SHR and WKY. The EC₅₀ for Ang II–induced PLD stimulation in SHR was 10-fold lower than the EC₅₀ in WKY, and both were inhibited by L-158,805, an AT₁ antagonist. Inhibitors of phosphoinositol-3-kinase and protein kinase C did not block Ang II–induced PLD activity in SHR and WKY PGSMCs. Catalytically-inactive constructs of PLD2 and RhoA, but not PLD1, ADP ribosylation factor 1 (ARF1), ARF6, or ADP ribosylation factor nucleotide exchange factor (ARNO) blocked Ang II–induced PLD activity in SHR and WKY PGSMCs. Brefeldin A completely blocked Ang II–induced PLD activity in SHR but only slightly reduced Ang II–induced PLD activity in WKY PGSMCs. Therefore, we conclude that in PGSMCs, the effect of Ang II on PLD activity is (1) greater in SHR; (2) mediated by AT₁ receptors signaling to PLD2; (3) transduced primarily by Rho proteins; and (4) inhibited in SHR by brefeldin A. (*Hypertension*. 2001; 37[part 2]:635-639.)

Key Words: angiotensin II ■ phospholipases ■ hypertension, experimental ■ RhoA ■ receptors, angiotensin ■ rats, inbred SHR ■ brefeldin A

Angiotensin II (Ang II) importantly contributes to the pathophysiology of hypertension in spontaneously hypertensive rats (SHR). Blood pressure in SHR is normalized by angiotensin-converting enzyme inhibitors,¹ AT₁ receptor antagonists,² and active immunization against renin.³ However, Ang II production is apparently not elevated in SHR, either systemically⁴ or locally.⁵ On the other hand, renal transplantation studies demonstrate that the kidneys mediate hypertension in SHR.^{6,7} A solid working hypothesis to explain these observations is the idea that hypertension in SHR may be due to enhanced renovascular sensitivity to Ang II.^{8,9} This hypothesis is supported by additional experimental data.¹⁰

The mechanism of enhanced renal sensitivity to Ang II is unclear but may involve multiple signaling pathways. Previous studies have demonstrated that Ang II–induced phospholipase D (PLD) activity is increased in thoracic smooth muscle cells from SHR compared with Wistar-Kyoto rats (WKY).¹¹ PLD generates phosphatidic acid, a lipid involved in the activation of mitogen-activated protein kinase (MAPK).^{12,13} Because MAPK activity is elevated in SHR vasculature¹⁴ and MAPK appears to be involved in vascular

smooth muscle cell contraction,^{15,16} it is possible that PLD mediates, in part, the enhanced renovascular response of SHR to Ang II.

We studied the mechanisms of PLD activation in cultured preglomerular microvascular smooth muscle cells (PGSMCs). Our initial studies demonstrate that Ang II activates PLD in PGSMCs and that this response is augmented in SHR PGSMCs vs those of WKY. Therefore, we investigated in SHR and WKY PGSMCs (1) the relative roles of Ang II receptor subtypes in stimulation of PLD; (2) the relative contributions of PLD isoforms to Ang II–dependent PLD activity; and (3) the signaling pathways that mediate Ang II–induced stimulation of PLD (Figure 1).

Methods

Drugs and Constructs Used

Ang II and PMA were obtained from Sigma Chemical Co. All other drugs were obtained from Calbiochem unless otherwise noted. L-158,809 and PD-123,319 were used to inhibit the AT₁ and AT₂ receptor, respectively. LY-294002 and wortmannin were used to inhibit phosphoinositol-3-kinase (PI3K). Ro-31-8425, Go-6983, and staurosporine were used to inhibit protein kinase C (PKC). The catalytically inactive (dominant negative [dn-]) mutants T31N

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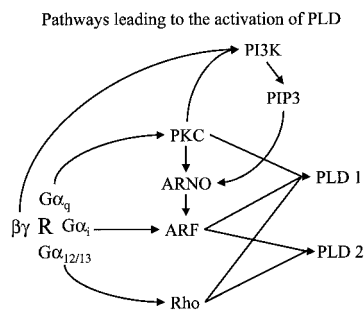


Figure 1. Pathways from a heterotrimeric G-coupled receptor (R) to PLD. Pathways are PI3K, PKC, ARNO, ARF, and RhoA.

ARF1, T27N ARF6, and T17N RhoA were used to inhibit specific G protein functions. E156K ADP ribosylation factor nucleotide exchange factor (ARNO), a dn-mutant of a specific ADP ribosylation factor (ARF) guanine nucleotide exchange factor (GEF), was also used. Catalytically inactive K898R PLD1 and K758R PLD2 were used to investigate PLD function. All constructs were previously subcloned as EGFP fusion proteins as described elsewhere.^{12,17}

Cell Culture

All cell culture reagents were obtained from Gibco BRL. PGSMCs were obtained from 13- to 15-week-old SHR and WKY as previously described.¹⁸ Briefly, 1% Fe₂O₃ DMEM was forcefully injected into isolated kidneys through the renal artery. The iron-loaded kidney was removed from the rat, and the cortex was minced and washed in a 1% collagenase IV solution. Vessels were plated with DMEM/F12 supplemented with 10% FCS and 20 U of penicillin-streptomycin. All experiments were conducted between passage 4 and 10 and in DMEM/F12 supplemented with 10% FBS.

PLD Assay

PGSMCs were serum-starved for ≥ 15 hours in 2 mL of DMEM/F12 with [³H]palmitate (5 μ Ci/mL). PGSMCs were incubated for 12 minutes in DMEM/F12 with 0.5% ethanol (EtOH medium). All antagonists used were included in the EtOH medium. Cells were stimulated with agonist for 20 minutes, washed with cold PBS, and scraped at 0°C. The formation of phosphatidylethanol was used to determine the activation of PLD as described previously.¹⁷

Transfection of PGSMCs

PGSMCs were grown to 50% to 75% confluence in 60-mm dishes and transfected with lipofectamine. The following day, the cells were washed and fresh media was added. Because all constructs used were subcloned as EGFP fusion proteins, the transfection efficiency was determined by the fraction of green fluorescent cells. Cells were serum-starved in the presence of 10 μ mol/L *n*-butyric acid to promote transcription of the EGFP fusion protein. Only plates that had >40% transfection efficiency were used.

Quantification of PLD mRNA

Total RNA was isolated by use of the Qiagen Rneasy mini kit protocol, and 10 μ L was used in the Clontech Advantage RT-for-PCR (reverse transcriptase-for-polymerase chain reaction) kit. The resulting cDNA was used to amplify PLD1, PLD2, and GAPDH as described previously.¹⁶ GAPDH was used as an internal control. PCR products were run on a 2% agarose gel stained with ethidium bromide. A digital picture was taken of the gel and analyzed with Molecular Analyst for Windows software.

Statistical Analysis

All data were compared by ANOVA and Fisher's least significant difference post hoc test. For individual comparisons, a *t* test was used to determine significance. Data points are indicated to be significant only if $P < 0.05$. Statistical analysis was conducted with the NCSS 2000 program. Dose-response curves were analyzed by use of the curve-fit routines of GraphPad Prism.

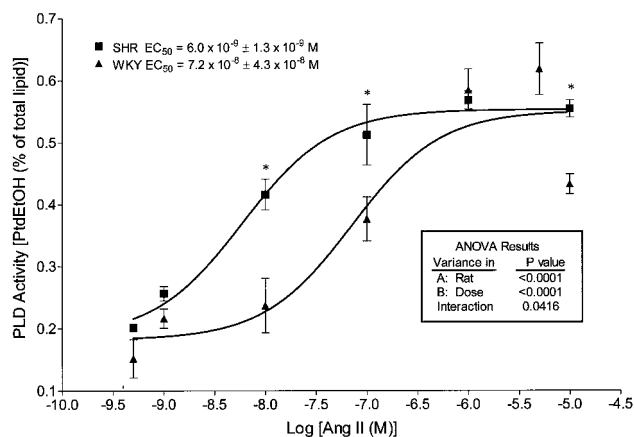


Figure 2. Ang II dose-response curve. Ang II was applied to SHR and WKY PGSMCs as described. Data represent mean \pm SEM. PtdEtOH indicates phosphatidylethanol. *Significantly different at $P < 0.05$.

Results

Ang II increased PLD activity in both SHR and WKY PGSMCs in a concentration-dependent fashion (Figure 2). However, EC₅₀ for Ang II in SHR cells (6.0×10^{-9} mol/L) was significantly less than that for WKY cells (7.2×10^{-8} mol/L). Two-factor ANOVA indicated a significant shift to the left of the SHR dose-response versus the WKY response curve (Figure 2). The AT₁ antagonist L-158,809 (1.5 nmol/L) inhibited Ang II-induced PLD activity in SHR and WKY PGSMCs by $92 \pm 9\%$ and $63 \pm 5\%$ respectively. The AT₂ specific antagonist PD-123,319 (15 nmol/L) did not decrease Ang II-dependent PLD activation in cells from either strain, which thus suggests that PLD activation is downstream of the AT₁ receptor in PGSMCs as previously shown in thoracic aortic smooth muscle cells.¹⁹

RT-PCR demonstrated the presence of all PLD isoforms in both SHR and WKY PGSMCs (Figure 3, top). Transfection of cells with catalytically inactive PLD2 blocked Ang II-induced PLD activity (Figure 3, bottom). However, transfection with the catalytically inactive PLD1 had no effects. These data suggest that PLD2 is the main signaling isoform in Ang II-mediated PLD activity.

Neither LY-294002 (7 μ mol/L) nor wortmannin (100 nmol/L) affected Ang II-induced PLD activity. Thus, PI3K activity is not essential for the activation of PLD by Ang II (Figure 4). Ro-31-8425 (390 nmol/L), Go-6983 (1 μ mol/L), and staurosporine (250 nmol/L) had no effects on Ang II-induced PLD activity, which suggests that the effects of Ang II on PLD are independent of PKC activity (Figure 5). However, a combination of Ro-31-8425 and Go-6983 slightly inhibited Ang II-induced PLD2 activity (Figure 5). PMA 500 nmol/L, a diacylglycerol analogue, served as a control for the PI3K and PKC inhibitors. All PKC inhibitors blocked PMA-induced PLD activity, and PI3K inhibitors slightly reduced PMA-induced PLD activity (data not shown).

Transfection of PGSMCs with T17N RhoA markedly reduced Ang II-induced PLD activity (Figure 6). In contrast, transfection of PGSMCs with E156K ARNO, T31N ARF1, and T26N ARF6 did not reduce Ang II-induced PLD activity

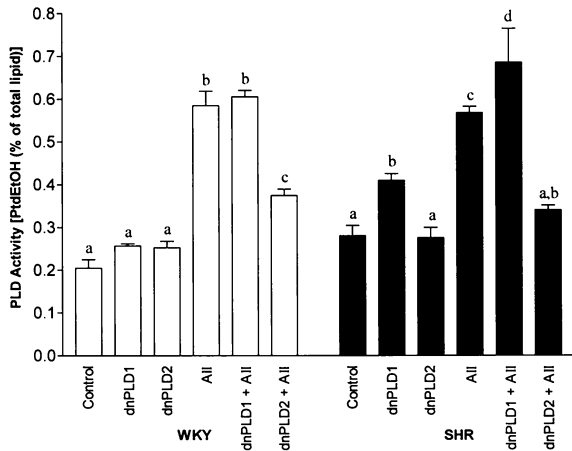
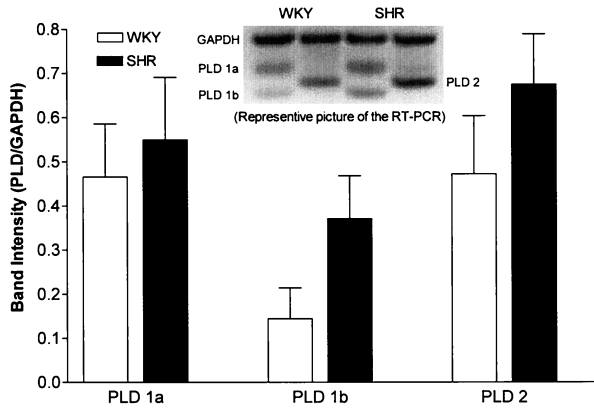


Figure 3. Expression of PLD isoforms in SHR and WKY PGSMCs. RT-PCR was used to measure the amount of PLD message (top). Catalytically inactive PLD1 and PLD2 were transfected into PGSMCs before stimulation with 1 $\mu\text{mol/L}$ Ang II (All, bottom). Data represent mean \pm SEM (n=3); lettered bars are significantly different at $P < 0.05$.

(Figure 6). Therefore, we conclude that activation of PLD by Ang II is primarily mediated by Rho proteins. In contrast to our previous data that show that ARF mediates Ang II-dependent PLD activity in A10 cells,¹⁷ ARF proteins did not

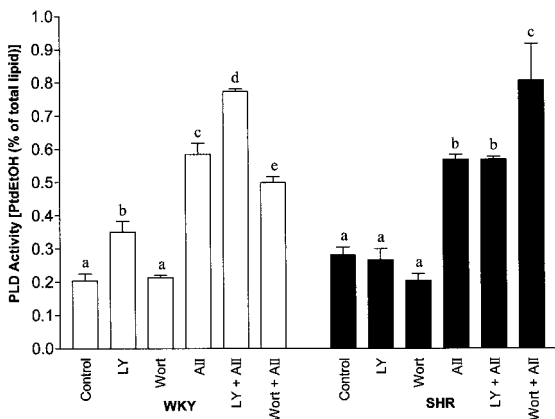


Figure 4. Effect of PI3K inhibitors on Ang II-mediated PLD2 activity. LY-294002 (LY; 7 $\mu\text{mol/L}$) and wortmannin (Wort; 100 nmol/L) were used to inhibit PI3K. Cells were stimulated with 1 $\mu\text{mol/L}$ Ang II. Data represent mean \pm SEM (n=3); lettered bars are significantly different at $P < 0.05$.

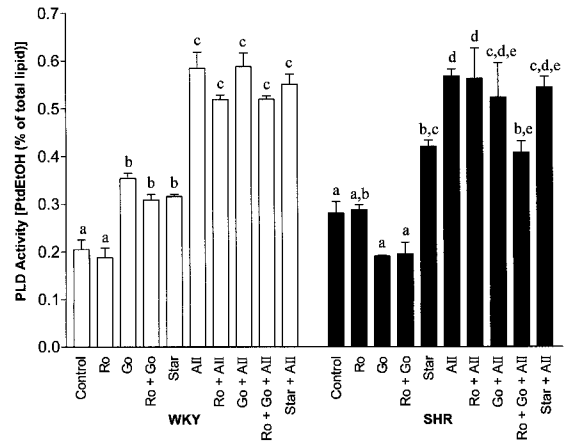


Figure 5. Effect of PKC inhibitors on Ang II-mediated PLD2 activity. Ro-31-8425 (Ro; 390 nmol/L), Go-6983 (Go; 1 $\mu\text{mol/L}$), and staurosporine (Star; 250 nmol/L) were used to inhibit PKC. Cells were stimulated with 1 $\mu\text{mol/L}$ Ang II. Data represent mean \pm SEM (n=3); lettered bars are significantly different at $P < 0.05$.

appear to play a significant role in the activation of PLD by Ang II in PGSMCs. However, BFA (50 $\mu\text{g/mL}$), an inhibitor of ARF activation, completely blocked Ang II-induced PLD activity in SHR PGSMCs but only reduced activity by 40 \pm 0.4% in WKY PGSMCs ($P < 0.5$ versus inhibition of SHR PGSMCs). This suggests that a target of BFA other than the classic BFA-sensitive ARF-GEFs is involved in Ang II-induced PLD activity in PGSMCs.

Discussion

PGSMCs modulate preglomerular vascular resistance and thus regulate renal blood flow, glomerular filtration rate, and, indirectly, renal sodium excretion.²⁰ Because renal function determines the long-term levels of arterial blood pressure,²¹

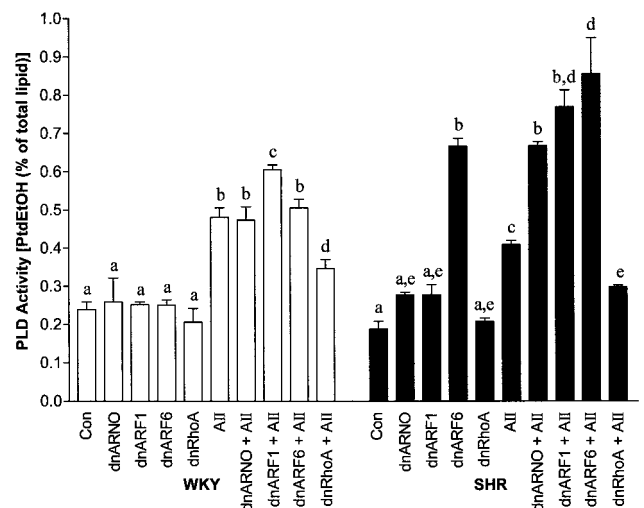


Figure 6. Effect of the inhibition of small G proteins of the ARF and Rho family on Ang II-mediated PLD2 activity. Dn-mutants were transfected into cells 24 hours before challenge 1 $\mu\text{mol/L}$ Ang II. Con indicates control cells transfected with GFP; A II, control cells (transfected with GFP) plus Ang II. Data represent mean \pm SEM (n=3); lettered bars are significantly different at $P < 0.05$.

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altered responses of PGSMCs to vasoactive factors are likely to be important in the pathophysiology of genetic hypertension. Several studies suggest that the preglomerular microcirculation is more responsive to Ang II in SHR versus WKY,^{8–10} and the basis for this hyperresponsiveness to Ang II is under intense investigation.

PLD is an important regulatory enzyme activated by several vasoactive agents, including Ang II and endothelin-1.¹⁷ Recent work has demonstrated that phosphatidic acid, the product of PLD-catalyzed hydrolysis of phosphatidylcholine, plays a central role in the regulation of the MAPK cascade.^{12,13} In A10 cells, a vascular smooth muscle cell line, the stimulation of MAPK phosphorylation by Ang II is inhibited in the absence of PLD activity, and addition of phosphatidic acid restores the effects of Ang II on MAPK phosphorylation.¹⁷ MAPK has been implicated in the phosphorylation of caldesmon, leading to increased smooth muscle contraction.¹⁵ Recent data indicate that MAPK inhibition (1) reduces Ang II-induced primary smooth muscle cell contraction and (2) normalizes the Ang II contractile response of SHR smooth muscle cells.¹⁶ Therefore, alterations in the regulation of PLD activity by vasoactive peptides may play an important role in the development of hypertension.

This study demonstrates that Ang II stimulates PLD activity in PGSMCs. We also show that SHR PGSMCs are significantly more sensitive to Ang II than WKY PGSMCs. To establish the biochemical basis for these differences, we examined in detail some aspects of the mechanism of signaling involved in the stimulation of PLD by Ang II. Previous studies on thoracic aorta vascular smooth muscle cells show that AT₁ receptors transduce signals to PLD.¹⁹ SHR and WKY PGSMCs are no different, because the selective AT₁ antagonist L-158,805 inhibited the effects of Ang II on PLD in both cases. This finding is consistent with our previous *in vivo* study that showed that the enhanced renovascular response to Ang II in the SHR kidney is mediated by AT₁ receptors.²²

We next examined the relative roles of PLD1 and PLD2. Semiquantitative PCR shows that SHR and WKY PGSMCs express similar amounts of PLD1 and PLD2. Transfection with catalytically inactive forms of PLD1 and PLD2 show that PLD2 is the main signaling isoform, thus confirming previous findings from our laboratory.^{12,17}

G protein-coupled receptors (GPCR) regulate PLD activity by several mechanisms.²³ For instance, GPCRs activate PI3K,²⁴ and phosphatidylinositol 4,5-bisphosphate or inositol 1,4,5-trisphosphate appears to be required for the activity of PLD.²⁵ Likewise, many GPCRs activate PKC, which has been shown to be an important regulator of PLD activity.²⁶ We have addressed the role of PI3K and PKC activation in the modulation of PLD activity by Ang II with specific inhibitors of these enzymes. Pharmacological blockade of PI3K and PKC does not attenuate Ang II-stimulated PLD activation.

Previous work from our laboratory has shown that ARF proteins are involved in the main pathway for regulation of PLD by Ang II and endothelin-1 receptors in A10 cells.¹⁷ We examined this pathway in PGSMCs by use of the ARF catalytically inactive mutants T31N ARF1 and T26N ARF6.

Our data show convincingly that neither of these mutants had any effect on Ang II-dependent PLD stimulation in PGSMCs. This evidence suggests that the main signaling pathway that leads to PLD activation is different in PGSMCs than in A10 cells. To confirm that ARF proteins were not involved in Ang II-dependent PLD activity, we transfected PGSMCs with a putative dn-mutant of ARNO, an ARF-GEF shown to be involved in agonist-dependent ARF activation.²⁷ As shown, this mutant did not alter the effects of Ang II on PLD stimulation.

Another mechanism by which GPCRs regulate PLD activation involves activation of Rho proteins by a mechanism mediated by the activation of G_{12/13}.²⁸ Because our work ruled out the involvement of PI3K, PKC, and ARF in Ang II-dependent PLD activation in PGSMCs, we tested the role of Rho by transfecting cells with T17N RhoA, a catalytically inactive mutant. Our data show that the expression of the mutant RhoA significantly attenuates the effects of Ang II on PLD. This finding is in marked contrast with our previous work on A10 cells, in which Rho did not play a significant role in PLD activation.¹⁷ The reasons why A10 cells, an established cell line, and PGSMCs, derived from primary culture, use different signaling pathways are not clear. However, these data indicate that the mechanisms that predominate in established tissue culture systems cannot necessarily be extrapolated to primary cells or *in vivo* conditions.

Finally, BFA, a toxin that blocks the activation of ARF proteins and inhibits PLD activation in other systems, including A10 cells,¹⁷ was also found to inhibit PLD activation in PGSMCs. This effect of BFA is not likely to be related to its effects on ARF activation, given that ARF catalytically inactive mutants did not reduce Ang II-mediated PLD activity. Furthermore, the effects of BFA were different in SHR and WKY PGSMCs; WKY PGSMCs are significantly less sensitive to BFA. This suggests that a BFA-sensitive pathway might be involved in the activation of PLD by Ang II in SHR. Elucidation of this pathway may provide a novel target for the development of a new antihypertensive drugs.

In summary, the present study demonstrates that Ang II stimulates PLD activity in PGSMCs and that the sensitivity of SHR, compared with WKY PGSMCs, is greater in this regard. The signaling pathway used by Ang II is AT₁ receptor to RhoA to PLD2. Elucidation of the proteins responsible for increased PLD activity in SHR may provide insight into the pathophysiology of genetic hypertension and reveal a novel target for a new class of antihypertensive drugs.

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