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AT_2 receptors cross talk with AT_1 receptors through a nitric oxide- and RhoA-dependent mechanism resulting in decreased phospholipase D activity

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Andresen, Bradley T., Kuntala Shome, Edwin K. Jackson, and Guillermo G. Romero. AT_2 receptors cross talk with AT_1 receptors through a nitric oxide- and RhoA-dependent mechanism resulting in decreased phospholipase D activity. *Am J Physiol Renal Physiol* 288: F763–F770, 2005. First published November 30, 2004; doi:10.1152/ajprenal.00323.2004.—ANG II activation of phospholipase D (PLD) is required for ERK and NAD(P)H oxidase activation, both of which are involved in hypertension. Previous findings demonstrate that ANG II stimulates PLD activity through AT_1 receptors in a RhoA-dependent mechanism. Additionally, endogenous AT_2 receptors in preglomerular smooth muscle cells attenuate ANG II-mediated PLD activity. In the present study, we examined the signal transduction mechanisms used by endogenous AT_2 receptors to modulate ANG II-induced PLD activity through either PLA_2 generation of lysophosphatidylethanolamine or $G\alpha_i$-mediated generation of nitric oxide (NO) and interaction with RhoA. Blockade of AT_2 receptors, $G\alpha_i$ and NO synthase, but not PLA_2, enhanced ANG II-mediated PLD activity in cells rich in, but not poor in, AT_2 receptors. Moreover, NO donors, a direct activator of guanylyl cyclase and a cGMP analog, but not lysophosphatidylethanolamine, inhibited ANG II-mediated PLD activity, whereas an inhibitor of guanylyl cyclase augmented ANG II-induced PLD activity. AT_2 receptor- and NO-mediated attenuation of ANG II-induced PLD activity was completely lost in cells transfected with S188A RhoA, which cannot be phosphorylated on serine 188. Therefore, our data indicate that AT_2 receptors activate $G\alpha_i$, subsequently stimulating NO synthase and leading to increased soluble guanylyl cyclase activity, generation of cGMP, and activation of a protein kinase, resulting in phosphorylation of RhoA on serine 188. Furthermore, because AT_2 receptors inhibit AT_1 receptor signaling to PLD via modulating RhoA activity, AT_2 receptor signaling can potentially regulate multiple vasoconstrictive signaling systems through inactivating RhoA.

cGMP; S188a RhoA; phosphorylation of RhoA; hypertension; vascular biology

EXHAUSTIVE STUDIES HAVE DEMONSTRATED that the spontaneously hypertensive rat (SHR) develops hypertension early in life by a mechanism that is dependent on the renin-angiotensin system, and inhibition of any component of this system normalizes the blood pressure of the SHR to that of its founder line and thus experimental control, the Wistar-Kyoto rat (WKY) (15, 30, 33). Furthermore, the renal vasculature generates the greatest contractile response to ANG II (24). Moreover, extensive studies utilizing kidney transplantation techniques indicate that the blood pressure phenotype is transferred with the kidney, thus indicating that the kidney is the organ responsible for hypertension (19). Not surprisingly, ANG II is a more potent renal vasoconstrictor in SHR than WKY rats (11).

Therefore, the wealth of data suggests that the primary defect in the SHR resides in ANG II-mediated signal transduction cascades related to contraction in the renal vasculature. In this regard, ANG II, acting via the angiotensin type 1 receptor (AT_1R), activates phospholipase D (PLD) more so in SHR compared with WKY preglomerular smooth muscle cells (PGSMCs) (3). PLD, an enzyme that hydrolyzes phosphatidylcholine, generating the lipid second messenger phosphatidic acid (PA), importantly contributes to the vascular effects of ANG II through PA-dependent activation of both the ERK (5) and NAD(P)H oxidase (36).

Regarding the role of PLD in ANG II-induced activation of the ERK pathway, PA is required for recruitment of Raf-1 to membranes (37). Our studies demonstrate that PLD activity is required for ANG II-mediated activation of MEK in both A10 smooth muscle cells (46) and PGSMCs (4). Therefore, stimulation of PLD activity by ANG II contributes to activation of the Raf-1/MEK/ERK cascade. Because MEK inhibitors, such as PD-98059, attenuate ANG II-mediated smooth muscle contraction (50) and increases in blood pressure (35), PLD activity can lead to increased vascular smooth muscle contraction and ultimately hypertension.

PLD is also involved in ANG II-mediated activation of NAD(P)H oxidase. In this regard, NAD(P)H oxidase is activated maximally by the synergistic actions of PA and diacylglycerol (36). ANG II generates free radicals via activation of a smooth muscle homolog of NAD(P)H oxidase (28). Moreover, PLD activity is required for ANG II-mediated generation of free radicals in human vascular smooth muscle (51). Furthermore, introduction of free radical scavengers reduces ANG II-induced vasoconstriction (29) and blood pressure in SHR (43). This suggests that PLD-mediated activation of NAD(P)H oxidase contributes to ANG II-induced vasoconstriction and hypertension.

Because of the important role of PLD in mediating the vascular effects of ANG II, our studies focus on the mechanisms regulating ANG II-mediated PLD activity in vascular smooth muscle cells. Our most recent results indicated that in cultured PGSMCs, the ANG II type 2 receptor (AT_2R) attenuates AT_1R-induced activation of PLD (6). Thus the overall effect of ANG II on PLD activation depends on the balance of AT_1Rs vs. AT_2Rs. However, the mechanism by which AT_2Rs attenuate AT_1R-mediated PLD activation is completely un-

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known, and the general purpose of the present study was to investigate this mechanism.

Physiological inhibition of PLD has been demonstrated to occur in plants through a phospholipase A_2 (PLA_2)-dependent mechanism. Specifically, PLA_2 generation of lysophosphatidylethanolamine (LPE) can inhibit PLD through an undefined mechanism (38). Because AT_2Rs have been shown to activate PLA (20, 25), AT_2R-mediated inhibition of PLD may be PLA and LPE dependent. Alternatively, RhoA, which we showed to be necessary for AT_1R-mediated PLD activity (3), can be inactivated through phosphorylation (13). It is well established that phosphorylation of RhoA on serine 188, by cyclic-nucleotide-dependent kinases (PKA and PKG) (13), promotes binding of Rho guanine nucleotide dissociation inhibitors (GDIs) to RhoA thus preventing RhoA from engaging RhoA interacting proteins (16). Moreover, it is known that endogenous AT_2R stimulation results in increased levels of renal nitric oxide (NO) and cGMP (47).

These facts prompted us to examine the hypothesis that AT_2Rs inhibit AT_1R-mediated PLD activity through a PLA_2- and LPE-dependent pathway or a NO- and cGMP-dependent mechanism that involves the inhibitory phosphorylation of RhoA on serine 188. This hypothesis was tested in cultured PGSMCs obtained from WKY rats and, in some experiments, SHR. The rational for using both WKY and SHR PGSMCs was that cultured WKY PGSMCs express sufficient AT_2Rs to interfere with AT_1R signaling, whereas SHR PGSMCs do not (6).

METHODS

Materials. The pharmacological agents used in this work were obtained from the following suppliers: Sigma (St. Louis, MO): ANG II, CGP-42112A, pertussis toxin, and N^5-(nitroamidino)-L-2,5-diaminopentanoic acid (NNA); Molecular Probes (Eugene, OR): *S*-nitroso-*N*-acetylpenicillamine (SNAP) and diethylamine nitric oxide (DEANO); Alexis (Carlsbad, CA): 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) and YC-1; Tocris (Ballwin, MO): 4-(4-octadecyl)-4-oxobenzenebutenoic acid (OBAA); Calbiochem (San Diego, CA): G418, 8-Br-cGMP, and botulinum toxin exoenzyme C3. [^{3}H]palmitate was obtained from New England Nuclear/PerkinElmer (Boston, MA). All lipids were obtained from Avanti Polar Lipids (Alabaster, AL), and all molecular biology enzymes were obtained from New England Biolabs (Beverly, MA). PD-123,319 was a gift from Merck. pEF C3 and the vector controls were received from Dr. J. Silvio Gutkind (National Institutes of Health/National Institute of Dental and Craniofacial Research). The S188A RhoA construct was generated as described below.

Cell culture and PLD assays. Cell culture reagents were obtained from Invitrogen/GIBCO BRL (Carlsbad, CA). PGSMCs were isolated from WKY and SHR from Taconic Farms (Germantown, NY) as described previously (3). The Institutional Animal Care and Use Committee approved all procedures. PLD assays were conducted as described previously (46) and all pharmacological agents were added to the assay media except for DEANO and SNAP, which were added immediately before ANG II. PLD assay data are expressed as the percentage of phosphatidylethanol (PtdEtOH) compared with total radiolabeled lipid. PGSMCs were transfected with Lipofectamine Plus from Invitrogen as per the manufacturer's directions, and the S188A RhoA-transfected cells were selected for with 200 μg/ml G418.

Cloning of WKY and SHR RhoA and generation of S188A RhoA. Total RNA was isolated from confluent 100-mm plates using the Rneasy Mini Kit (Qiagen, Valencia, CA). Ten micrograms of total RNA were used in a Clontech Advantage RT-for-PCR Kit (Palo Alto, CA). The resulting cDNA was amplified using Deep Vent polymerase (Invitrogen) with the following primers: 5′-GACGAATTCA-ATGGCTGCCATCAGGAAG-3′ and 5′-GGATCCTAC-TGAGGCTGCGTTCACAAG-3′. The product was inserted into the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). The S188A RhoA mutant was generated from WKY RhoA by PCR with the following primers: 5′-TACTCGAGCT-ATGGCTGCCATCAGGAAGAA-3′ and 5′-ATCTGCAG-TCACAAGATGAGGCACCCCG(C)CTTTTT-3′. An A-to-C mutation was introduced in the reverse primer at (C) to replace the endogenous serine at position 188 with alanine. The PCR products were ligated into pEGFP-C1 (Clontech), and the resulting colonies were screened and sequenced.

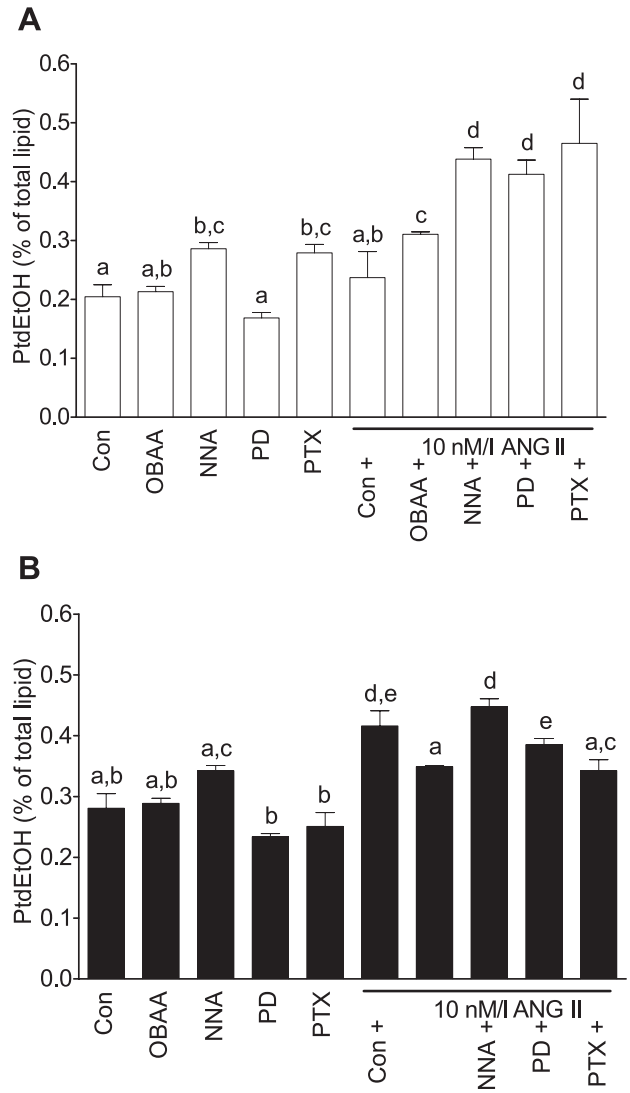

Fig. 1. Angiotensin type 2 receptors (AT_2) inhibit AT_1 receptor-mediated phospholipase D (PLD) activation through $G\alpha_i$ activation of nitric oxide synthase (NOS) in Wistar-Kyoto rat (WKY) but not spontaneously hypertensive rat (SHR) preglomerular smooth muscle cells (PGSMCs). *A*: 10 nM ANG II-mediated PLD activity is significantly increased by 1 μM N^5-(nitroamidino)-L-2,5-diaminopentanoic acid (NNA), 15 nM PD-123,319 (PD), and 1 ng/ml pertussis toxin (PTX) in WKY PGSMCs (open bars). Seven-hundred nanomolar 4-(4-octadecyl)-4-oxobenzenebutenoic acid (OBAA) does not increase 10 nM ANG II-mediated PLD activity to the extent of NNA, PD, and PTX in WKY PGSMCs. *B*: 10 nM ANG II-mediated PLD activity is not increased by 1 μM NNA, 15 nM PD, 1 ng/ml PTX, or 700 nM OBAA in SHR PGSMCs (filled bars). Data are expressed as means ± SE, $n = 3$; bars with different letters are significantly different ($P < 0.05$) as determined by ANOVA with Fisher's least significant difference (LSD) as a post hoc test.

Data and statistical analysis. The following error propagation formulas were applied. If f and g are two means and fe and ge are their respective error, then the error for f/g is $[fe \times g - f \times ge]/g^2$ and the error for $f \pm g$ is fe+ge. For multiple comparisons, the data were analyzed by ANOVA with Fisher's least significant difference post hoc test; for individual comparisons, a *t*-test was used to determine significance. Statistical analysis was conducted using the NCSS 2000 software package (Kaysville, UT). Dose-response curves were analyzed using the curve fit routines of GraphPad Prism (San Diego, CA).

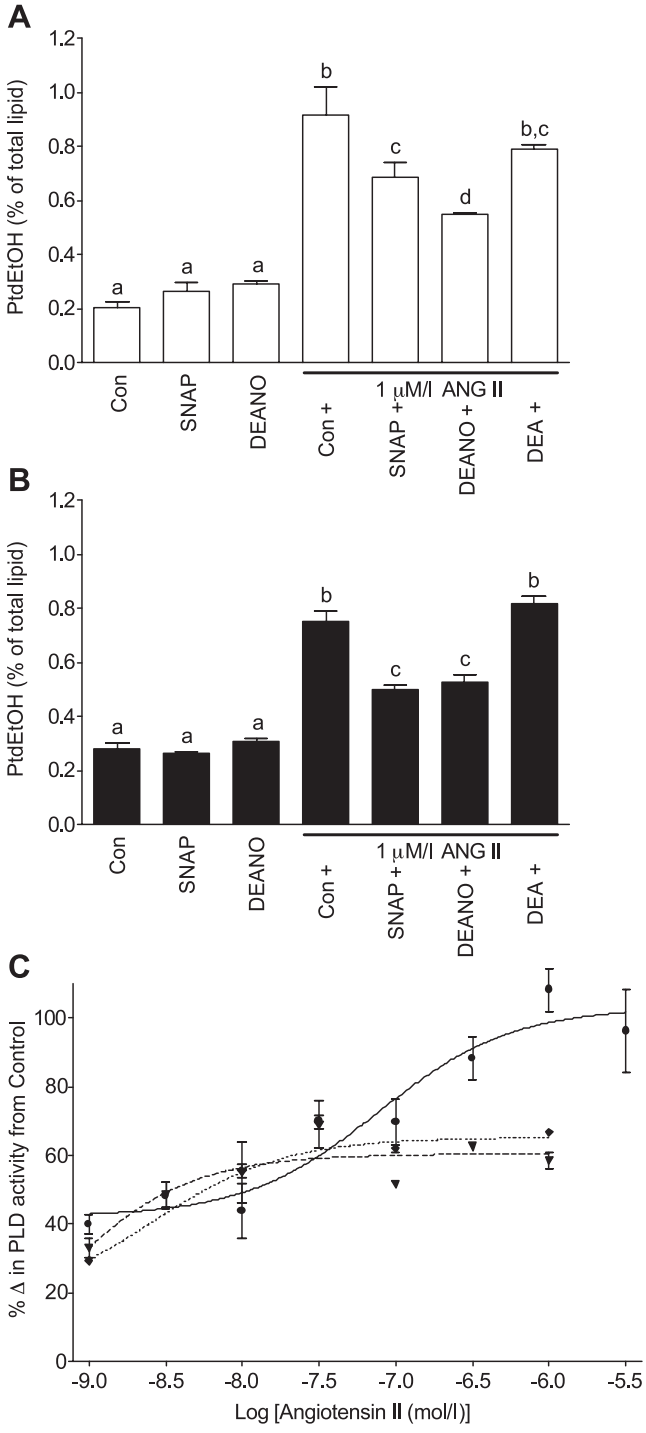

Fig. 2. NO attenuates ANG II-mediated PLD activity. *A*: 1 μM ANG II-mediated PLD activity is significantly attenuated by 200 μM structurally distinct NO donors *S*-nitroso-*N*-acetylpenicillamine (SNAP) and diethylamine nitric oxide (DEANO) in WKY PGSMCs (open bars). Two hundred micromolar DEA, DEANO that was allowed to incubate overnight at 37°C thus losing most of the NO, does not attenuate ANG II-mediated PLD activity to the same extent as DEANO. *B*: 1 μM ANG II-mediated PLD activity is significantly attenuated by 200 μM SNAP and DEANO in SHR PGSMCs (filled bars), and 200 μM DEA has no effect on 1 μM ANG II-mediated PLD activity. Data are expressed as means ± SE, $n = 3$; bars with different letters are significantly different ($P < 0.05$) as determined by ANOVA with Fisher's LSD as a post hoc test. *C*: ANG II activates PLD resulting in a maximal activity of approximately 2-fold over basal in WKY PGSMCs (●, solid line). Addition of 100 nM CGP-42112A (▼, dashed line) or NO from 200 μM DEANO (◆, dotted line) significantly reduces, as determined by a 2-factor ANOVA, the efficacy of ANG II-mediated PLD activity by ~40%. Data for the concentration-response curves are expressed as means ± SE, $n \geq 3$.

RESULTS

NOS and NO mediate AT_2R inhibition of AT_1R-induced PLD activity. We reported that WKY PGSMCs contain sufficient AT_2R expression to interfere with AT_1R signaling to PLD and that 10 nM ANG II-mediated PLD activity is enhanced by inhibiting the AT_2Rs in WKY but not SHR PGSMCs (6). As shown in Fig. 1, inhibition of NOS with 1 μM NNA, $G\alpha_i$ signaling with 100 ng/ml pertussis toxin, and antagonism of AT_2Rs with 15 nM PD-123,319 enhanced the ability of 10 nM ANG II-mediated PLD activity to a similar extent in WKY PGSMCs. However, in SHR PGSMCs none of the aforementioned inhibitors significantly enhanced 10 nM ANG II-mediated PLD activity. Inhibition of PLA_2, another proposed signal transduction mechanism for the AT_2Rs (22, 25), with 700 nM OBAA, used at a concentration shown to inhibit PLA_2 (26), had no effect on ANG II-mediated PLD activity in either WKY or SHR PGSMCs. These data indicate that AT_2R stimulation of NOS through $G\alpha_i$ proteins mediates AT_2R inhibition of AT_1R-mediated PLD activity in WKY PGSMCs.

Furthermore, addition of 200 μM concentrations of the NO donors SNAP or DEANO significantly decreased the ability of 1 μM ANG II, a concentration previously shown to give a maximal response in PGSMCs (3), to activate PLD in both WKY and SHR PGSMCs (Fig. 2*A*). DEA was generated from DEANO by preincubating 200 μM DEANO at 37°C in serum-free media overnight to release all NO from DEANO. The effect of DEA on ANG II-mediated PLD activity was significantly smaller than that of DEANO, indicating that the inhibition from DEANO is due to NO not the donor molecule. The addition of LPE did not inhibit ANG II-mediated PLD activity in WKY or SHR PGSMCs (data not shown), thus confirming that the AT_2R is not acting through a PLA- and LPE-dependent mechanism.

The effect of NO in both WKY and SHR PGSMCs supports our earlier observations that the primary difference between ANG II-mediated PLD regulation is the imbalance of AT_1Rs and AT_2Rs (6). Moreover, the ANG II dose-response curve generated in the presence of 200 μM DEANO is indistinguishable from the concentration-response curve generated in the presence of 100 nM of the AT_2R agonist CGP-42112A (Fig. 2*B*). These data indicate that the AT_2R inhibits AT_1R-mediated PLD activity through an NO-dependent mechanism.

cGMP mediates AT_2R inhibition of AT_1R-induced PLD activity. The activation of sGC is one the most common modes of NO action. To examine if sGC activation is sufficient to inhibit

ANG II-mediated PLD activity, YC-1, a NO-independent activator of sGC (23), was used in WKY PGSMCs. Ten micromolar YC-1 inhibited 1 μM ANG II-mediated PLD activity (Fig. 3). Simultaneous treatment of WKY PGSMCs with 200 μM DEANO and 10 μM YC-1 inhibited ANG II-mediated PLD activity to the same level as YC-1 alone and to a greater extent than DEANO alone, although this latter difference was not statistically significant. Thus the data indicate that NO-mediated sGC activation leads to inhibition of ANG II-mediated PLD activity.

Ten micromolar sGC inhibitor ODQ (18) potentiated the ability of 10 nM ANG II to stimulate PLD in WKY PGSMCs (Fig. 4*A*). The magnitude of the effect of ODQ on ANG II-induced PLD activation in WKY PGSMCs was similar to that observed for PD-123,319. Additionally, 100 μM 8-Br-cGMP, a cell-permeable cGMP analog, inhibited 1 μM ANG II-medaited PLD activity in WKY PGSMCs (Fig. 4*B*). These data confirm that the AT_2R inhibits AT_1R-mediated PLD activity through a sGC/cGMP-dependent mechanism.

Signal convergence at RhoA. To confirm our previous findings that RhoA is necessary for ANG II-mediated PLD activity in WKY PGSMCs, we used botulinum toxin exoenzyme C3 that inhibits RhoA activation (2). In WKY PGSMCs scrape loaded with the C3 protein (Fig. 5A) and expressing C3 cDNA (Fig. 5*B*), 1 μM ANG II failed to activate PLD, whereas 1 μM ANG II activated PLD in control-scraped cells (Fig. 5*A*) and the vector control (Fig. 5*B*). Therefore, functional RhoA is required for ANG II-mediated PLD activity in WKY PGSMCs; thus physiological inhibition of RhoA may inhibit ANG II-mediated PLD activity. To test whether AT_2R-mediated inhibition of RhoA, through phosphorylation, inhibits ANG II-mediated PLD activity, WKY PGSMCs were stably transfected with S188A RhoA. The S188A RhoA construct generates a mutant RhoA that cannot be phosphorylated and therefore does not interact with GDIs (16). Thus S188A RhoA is only insensitive to the physiological inhibition of RhoA mediated by phosphorylation and subsequent GDI interaction and functions as a wild-type in all other aspects (13).

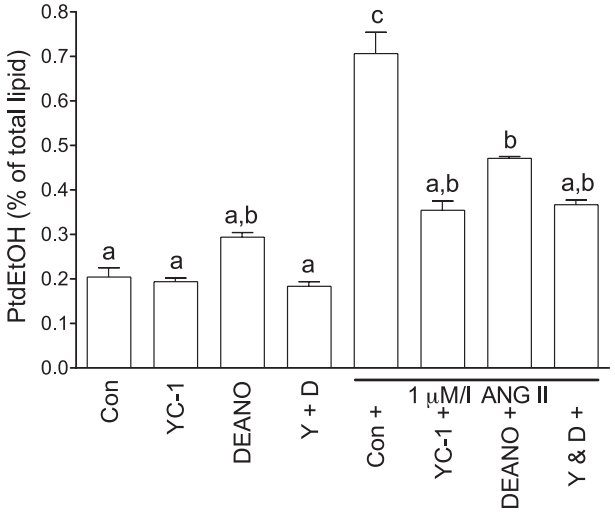

Fig. 3. Activation of sGC inhibits ANG II-mediated PLD activity. One micromolar ANG II-mediated PLD activity is significantly attenuated by 10 μM NO-independent stimulant of sGC, YC-1, in WKY PGSMCs. Ten micromolar YC-1 and 200 μM DEANO in combination (Y & D) does not further attenuate ANG II-mediated PLD activity compared with YC-1 or DEANO alone. Data are expressed as means ± SE, $n = 3$; bars with different letters are significantly different ($P < 0.05$) as determined by ANOVA with Fisher's LSD as a post hoc test.

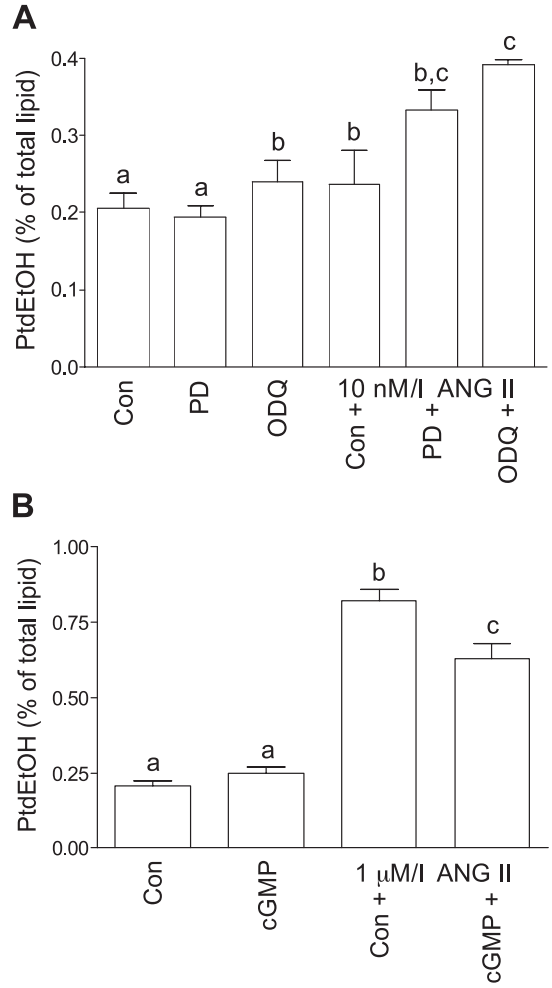

Fig. 4. sGC generation of cGMP inhibits ANG II-mediated PLD activity. *A*: 10 nM ANG II-mediated PLD activity is significantly enhanced by 10 μM of the sGC inhibitor 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) similarly to 15 nM PD in WKY PGSMCs. *B*: addition of 100 μM 8-Br-cGMP (cGMP) attenuates 1 μM ANG II-mediated PLD activity in WKY PGSMCs. Data are expressed as means ± SE, $n = 3$; bars with different letters are significantly different ($P < 0.05$) as determined by ANOVA with Fisher's LSD as a post hoc test.

In WKY PGSMCs expressing S188A, RhoA ANG II-mediated PLD activity was not enhanced by 15 nM PD-123,319 (Fig. 6*A*) and was not inhibited by 100 nM CGP-42112A (Fig. 6*B*). In contrast, PD-123,319 increased and CGP-42112A decreased ANG II-mediated PLD activity in control WKY PGSMCs. Additionally, 200 μM DEANO did not inhibit ANG II-mediated PLD activity in WKY PGSMCs expressing S188A RhoA yet did inhibit ANG II-mediated PLD activity in control cells (Fig. 7*A*). Furthermore, 200 μM DEANO had no effect on the ANG II-mediated PLD activity concentration-response curve in S188A RhoA-expressing cells (Fig. 7*B*). As shown in Fig. 7*C*, the concentration-response curve for ANG II-mediated PLD activity in S188A RhoA cells was shifted to the left and more closely resembled the concentration-response curve of WKY PGSMCs treated with PD-123,319 and that of SHR PGSMCs than control WKY PGSMCs. Consequently, the

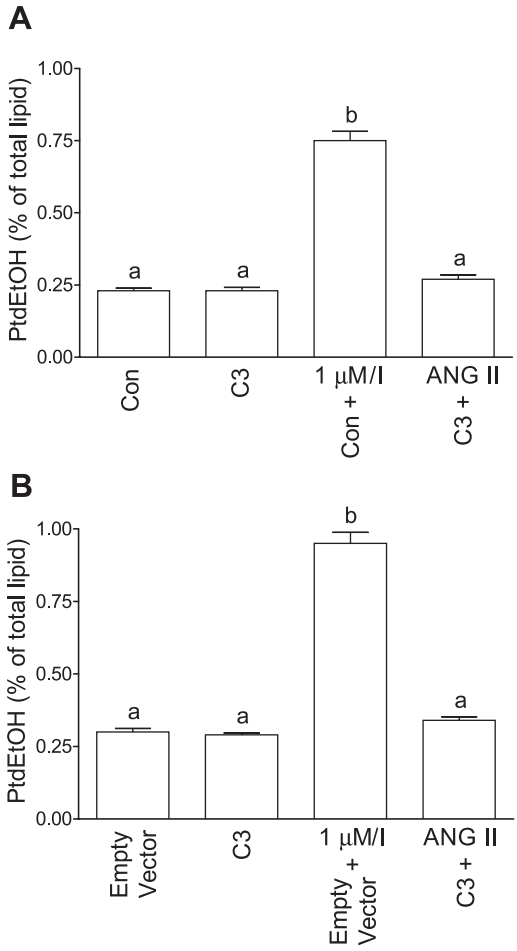

Fig. 5. Exotoxin C3 inhibits ANG II-mediated PLD activity. *A*: WKY PGSMCs scrape loaded with 5 μg/ml C3 exoenzyme (C3) do not respond to 1 μM ANG II, whereas cells scraped and loaded with buffer (Con) do respond to ANG II. *B*: similarly, transfection of C3 cDNA, but not empty vector, inhibits ANG II-mediated PLD activity. Data are expressed as means ± SE, $n = 3$; bars with different letters are significantly different ($P < 0.05$) as determined by ANOVA with Fisher's LSD as a post hoc test.

EC_{50} values for WKY PGSMCs expressing S188A RhoA are comparable to those of WKY cells treated with PD-123,319 and SHR PGSMCs (Table 1).

We also compared the sequences of RhoA isolated from WKY and SHR PGSMCs. No differences were found (GenBank accession nos. AY026068 and AY026069). In fact, we found only one difference from the previous rat GenBank entry, D84477, and this apparent mutation is silent.

DISCUSSION

AT_2Rs appear to participate importantly in vascular biology. In this regard, AT_2Rs are present in the vasculature and induce vasorelaxation in vivo (47), and multiple recent reports indicate that the vascular functions of AT_2Rs are unmasked when AT_1Rs are inhibited (9, 12, 53). Moreover, endogenous AT_2Rs are necessary for proper bradykinin-mediated vasodilation (8), and AT_2Rs cause NO-induced vasorelaxation (12, 47).

Data from ANG II receptor knockout mice confirm that there is physiological cross talk, at the level of vascular tone, between AT_1Rs and AT_2Rs and that AT_2Rs are expressed in the adult mouse (1, 48). These data, in conjunction with the pharmacological data, suggest that changes in ANG II receptor levels affect ANG II-mediated signal transduction resulting in alterations in ANG II-mediated blood pressure responses. Our recent findings demonstrate that the ratio of AT_1Rs to AT_2Rs in cultured WKY PGSMCs is approximately one but is greater in SHR PGSMCs due both to greater expression of AT_1Rs and reduced expression of AT_2Rs (6). We also found that the imbalance in receptor numbers could account for the increased potency of ANG II, with regard to increasing PLD activity, in SHR PGSMCs. Thus WKY PGSMCs provide a cell culture system for examining endogenous AT_2R signaling and SHR PGSMCs provide an unresponsive control group.

In WKY PGSMCs, blockade of AT_2Rs augments ANG II-mediated PLD activity, but this is not seen in SHR PGSMCs (6). These data suggest that AT_2Rs suppress ANG II-mediated PLD activity. The results of the present study are consistent with AT_2R regulation of AT_1R-mediated PLD activation by the following mechanism: $G\alpha_i$-coupled AT_2Rs lead to NOS-dependent generation of NO that then stimulates sGC to produce cGMP leading to reduced AT_1R-mediated PLD activation through the inhibitory phosphorylation of RhoA.

Our proposed mechanism for the interaction between AT_2Rs and AT_1Rs on PLD activity is supported by the results of the current study. In WKY PGSMCs, but not in SHR PGSMCs, blockade of $G\alpha_i$ and NOS has similar effects on ANG II-mediated PLD activity as does antagonism of AT_2Rs, which supports previous work indicating that AT_2Rs are coupled to

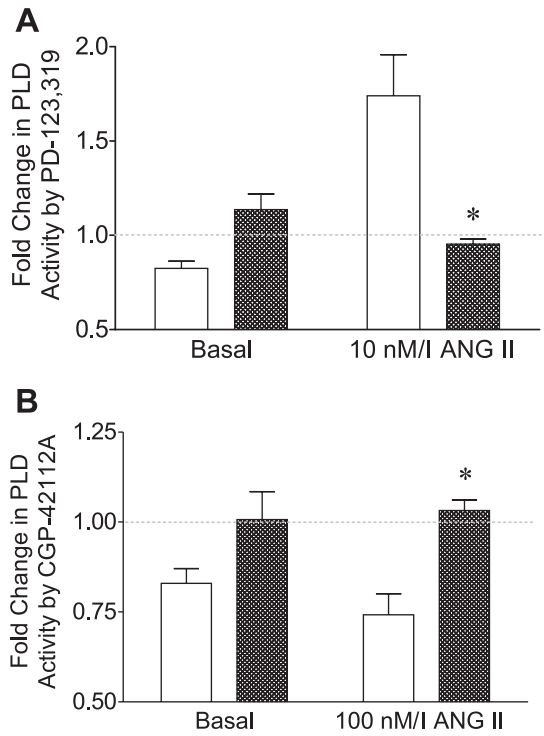

Fig. 6. WKY PGSMCs stably expressing S188A RhoA no longer respond to AT_2 receptor-specific drugs. *A*: 15 nM PD increases ANG II-mediated PLD activity in WKY (open bars) PGSMCs but has no effect in WKY PGSMCs expressing S188A RhoA (gray bars). *B*: 100 nM CGP-42112A fails to inhibit ANG II-mediated PLD activity in the presence of S188A RhoA (gray bars). Data are expressed as means ± SE of AT_2 receptor drug/control, $n = 3$; the gray dotted line represents expected values if the AT_2 receptor drug has no effect. *S188A RhoA-expressing cells are significantly different from control via Student's *t*-test from control ($P < 0.05$).

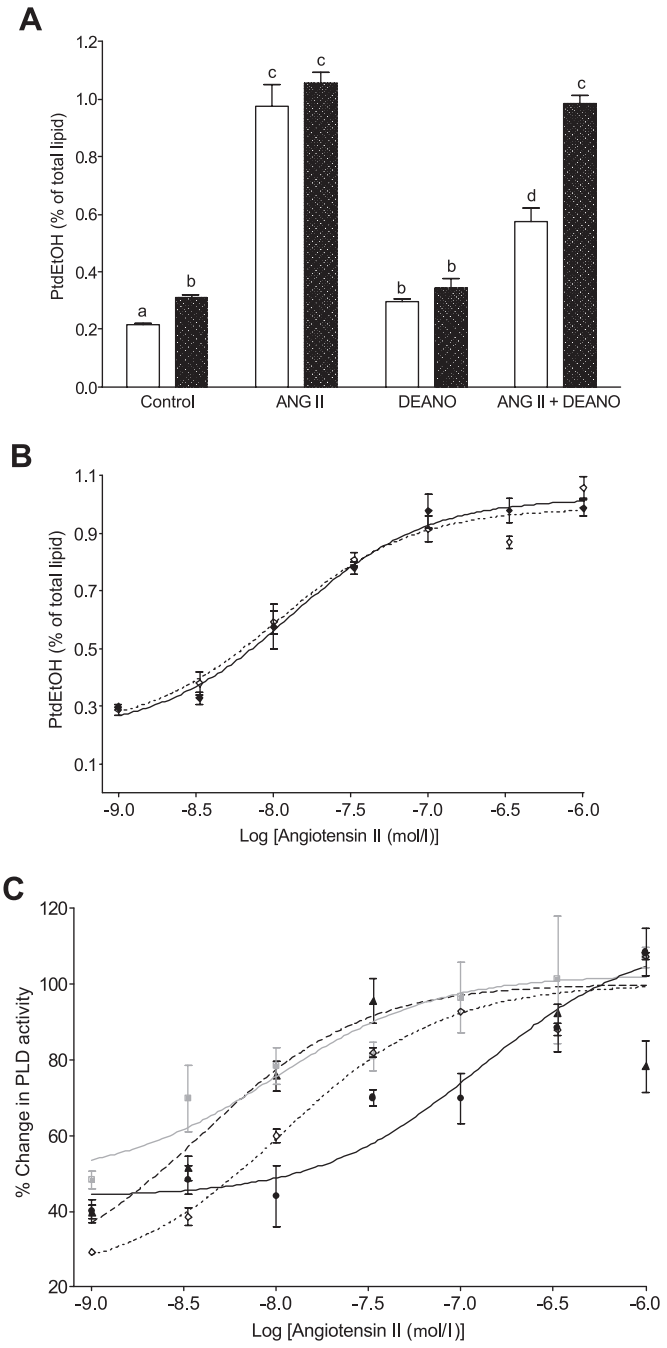

Fig. 7. WKY S188A RhoA-expressing cells do not respond to NO and behave more like SHR than WKY PGSMCs. *A*: 200 μM DEANO inhibits ANG II-mediated PLD activity in WKY PGSMCs (open bars); however, stable expression of S188A RhoA eliminates the effects DEANO has on ANG II-mediated PLD activity (cross-hatched bars). *B*: furthermore, expression of S188A RhoA (◇, dotted line) eliminates the 200 μM DEANO/NO-mediated (◆, solid line) decrease in efficacy. Data are expressed as means ± SE, $n = 3$; bars with different letters are significantly different ($P < 0.05$) as determined by ANOVA with Fisher's LSD as a post hoc test. *C*: moreover, expression of S188A RhoA (◇, dotted line) shifts the ANG II-mediated PLD activity concentration-response curve leftwards in such a way that it more closely resembles the SHR curve (■, gray line) and the WKY treated with 15 nM PD curve (▲, dashed line) than the control WKY curve (●, black line). Data are expressed as means ± SE, $n \geq 3$.

Table 1. *EC_{50} values for ANG II-mediated PLD activity in SHR, WKY, and WKY PGSMCs expressing S188A RhoA*

	WKY	SHR	WKY + PD-123,319	WKY S188A RhoA
Log EC_{50}, mol/l	−6.895	−8.001	−8.391	−7.960
Log standard error	0.272	0.302	0.677	0.183

PLD, phospholipase D; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; PGSMCs, preglomerular smooth muscle cells.

$G\alpha_i$ (21) and can activate NOS (10). Additionally, in WKY PGSMCs NO mimics the effects of stimulating AT_2Rs, thus confirming the data obtained with NNA. Due to bypassing AT_2Rs, NO also decreases ANG II-mediated PLD activity in SHR PGSMCs. The effect of NO in SHR PGSMCs further confirms our hypothesis that the overriding difference between WKY and SHR PGSMCs with regard to ANG II-mediated PLD activity is the altered ratio of AT_1 to AT_2 receptors.

Addition of YC-1 mimics the effects of DEANO, indicating that NO is inhibiting ANG II-mediated PLD activity through sGC. Furthermore, YC-1 and DEANO are not additive suggesting that NO acts only through activation of sGC to inhibit ANG II-mediated PLD activity. Moreover, in WKY PGSMCs, blockade of sGC mimics the effects of antagonism of AT_2Rs, thus confirming that AT_2R-mediated inhibition of AT_1R-mediated PLD activity requires sGC activity. Likewise, a cell-membrane permeant analog of cGMP reduces ANG II-mediated PLD activity, a result consistent with the involvement of sGC in the proposed signal transduction mechanism.

Consistent with our previous findings, RhoA activity is required for ANG II-mediated PLD activity as demonstrated by the inhibition seen with exotoxin C3. To access the role of phosphorylation of RhoA, we generated a mutant of RhoA that lacks the inhibitory phosphorylation site serine 188, S188A RhoA, and thus cannot be regulated through GDI-dependent mechanisms. In WKY PGSMCs stably expressing S188A RhoA, the ability of PD-123,319 to enhance ANG II-mediated PLD activity is blocked and the ability of CGP-42112A to attenuate ANG II-mediated PLD activity is also blocked. These results indicate a critical role for RhoA in both the signal transduction mechanism for AT_1R-medaited activation of PLD and AT_2R-mediated inhibition of PLD activity.

WKY PGSMCs expressing S188A RhoA failed to respond to NO. ANG II concentration-response curves in WKY PGSMCs expressing S188A RhoA more closely resemble SHR and PD-123,319-treated WKY PGSMCs. This supports the notion that AT_2R-mediated inhibition of PLD activity is mainly due to the inhibitory actions of the AT_2R on RhoA. Moreover, these data indicate that RhoA is the point of negative cross talk between AT_1Rs and AT_2Rs in regard to PLD activity. Importantly, increased levels of active RhoA have been associated with hypertension in SHR (44, 45). Because no mutations/polymorphisms were found in SHR RhoA, with respect to WKY RhoA, the increased RhoA activity in SHR must arise from aberrant regulation of RhoA.

RhoA activity is controlled by three mechanisms: *1*) activation by guanine nucleotide exchange factors (GEFs), *2*) inactivation by GTPase activating proteins (GAPs), and *3*) inhibition by GDIs. Increased activity of GEFs can lead to increased RhoA activity; similarly, a decrease in activity of GAPs will

lead to prolonged RhoA activation and increased signaling. GDIs interact with guanine-nucleotide-bound RhoA (16), sequester the protein, and relocate RhoA to the cytosol while masking the GEF and effector binding sites (27). Thus AT_2R-mediated phosphorylation of RhoA on serine 188 decreases the availability of RhoA for AT_1R-mediated activation of PLD, and consequently PLD activity is inhibited. Because the SHR PGSMCs have an imbalance in ANG II receptors that favors AT_1Rs (6), RhoA is more active in SHR compared with WKY PGSMCs (TM Seasholtz and EK Jackson, unpublished observations). Therefore, ANG II-mediated RhoA-dependent signaling mechanisms, such as PLD, are increased in SHR compared with WKY (3, 17).

Taken together, our data support our proposed mechanism by which the AT_2R inhibits ANG II-mediated PLD activity. However, the identity of the cyclic nucleotide-dependent kinase involved in the pathway remains unknown. Because cGMP is a known activator of PKG (49) and PKG phosphorylates RhoA at serine 188 (13, 40, 42), the most compelling conclusion is that PKG mediates phosphorylation of RhoA; however, NO/cGMP can lead to the activation of PKA (31, 39, 52), which can also phosphorylate RhoA at the same position (13, 14). Therefore, activation of either kinase could result in the same effect on ANG II-mediated PLD activity.

Although inhibition of AT_2Rs with PD-123,319 and pertussis toxin increases ANG II-induced PLD activation, it is conceivable, although unlikely, that other receptors activated by ANG II mediate, in part, the observed reduction in PLD activation. The experiments presented here were conducted to check each step in the signaling pathway for AT_2Rs using an inhibitor and activator of each proposed signaling molecule, including the use of an AT_2R antagonist and agonist, which gave opposite results, suggesting that the observed affects can be attributed to the AT_2R. However, if there is an alternative ANG II receptor mediating the affects attributed to the AT_2R, then the signal transduction cascade by which the alternate receptor interacts with ANG II-mediated PLD activity is the same as we have concluded for the AT_2R.

These data and conclusions may have implications regarding the treatment of hypertension. Angiotensin-converting enzyme (ACE) inhibitors and AT_1R blockers (ARBs) are commonly prescribed for patients with hypertension. It is unknown whether it is better to block angiotensin signaling in general via use of ACE inhibitors or just the "pathological signaling" of the AT_1Rs with ARBs. ARBs would be expected to attenuate RhoA activity in the renal microcirculation more so than would ACE inhibitors due to the effect on AT_2Rs. Because RhoA, through Rho kinases, is involved in smooth muscle contraction, inhibition of RhoA through phosphorylation inhibits contraction (34). Additionally, RhoA activates PLD, which is involved in stimulating NAD(P)H and ERK pathways, therefore inhibiting RhoA may reduce smooth muscle contraction through multiple mechanisms. Moreover, AT_2R-mediated inactivation of RhoA may potentially interfere with other activators of RhoA that have been proposed to contribute to hypertension, such as urotensin II (41), endothelin (32), and norepinephrine (7). Therefore, ARBs have a theoretical advantage over ACE inhibitors in that they inhibit the AT_1R, consequently inhibiting the vasoconstrictive actions of ANG II while allowing ANG II to stimulate endogenous AT_2Rs, thus interfering with general molecular mechanisms of vascular contraction. In addition to avoiding excessive vascular contraction, inhibition of RhoA and consequently PLD should reduce the production of free radicals and cellular proliferation within the renal microcirculation.

In summary, AT_2Rs inhibit AT_1R-mediated PLD activation through a NO/cGMP-dependent mechanism most likely mediated by phosphorylation of RhoA at serine 188. Furthermore, this shows that the AT_2R does not act as a mere antagonist to AT_1R-mediated functions but as an independent receptor with important vascular signaling characteristics. These results imply that selective stimulation of AT_2Rs may significantly contribute to the pharmacology of ARBs in the renal microcirculation.

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DISCLOSURES

There are no conflicts of interest or disclosures relevant to these studies with any of the authors.

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