AT₂ Receptors Attenuate AT₁ Receptor-Induced Phospholipase D Activation in Vascular Smooth Muscle Cells

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AT2R Attenuates AT1R–Induced PLD Activity

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D) Abbreviations:
SHR = Spontaneously hypertensive rat
WKY = Wistar-Kyoto rat
PGSMCs = Preglomerular vascular smooth muscle cells
PLD = Phospholipase D
AT1 = Angiotensin II receptor, type I
AT2 = Angiotensin II receptor, type II

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AT1 Angiotensin Receptors, AT2 Angiotensin Receptors, Phospholipase D, Angiotensin II, and Signal Transduction.
Abstract

Previous studies indicate that AT₁ receptor-induced activation of phospholipase D (PLD) may importantly contribute to vascular hypertrophy, injury and contraction. However, the role of AT₂ receptors in regulating AT₁ receptor-induced PLD activation is unknown. In this study we identified angiotensin II receptors on cultured preglomerular vascular smooth muscle cells (PGSMCs) from spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) by RT-PCR and binding assays and examined their functional effects on angiotensin II-mediated PLD activity. Both RT-PCR and binding indicated that cultured SHR and WKY PGSMCs expressed AT₁ and AT₂ receptors, and the combined total of AT₁ and AT₂ receptors was similar between the strains. However, the number of AT₁ and AT₂ receptors differed between SHR and WKY PGSMCs in so much as the ratio of AT₁-to-AT₂ receptors was approximately 1-to-1 and 3-to-1 in WKY and SHR PGSMCs, respectively. As previously reported, angiotensin II more potently activated PLD in SHR PGSMCs (SHR EC₅₀ = 4 nM; WKY EC₅₀ = 47 nM). Addition of an AT₂ receptor-specific antagonist or agonist shifted the angiotensin II-mediated PLD concentration-response curve of WKY PGSMCs in a manner consistent with AT₂ receptors producing an inhibitory signal. In contrast, in SHR little change was observed. Our findings indicate that the ratio of AT₁-to-AT₂ receptors in vascular smooth muscle cells may be a determinant of the net effects of angiotensin II on PLD activity due to AT₂-dependent inhibition of AT₁-mediated PLD activity. Furthermore, cultured WKY PGSMCs provide an excellent model system to study endogenous AT₂ receptor signal transduction.
Angiotensin II (Ang II) signal transduction is initiated by two G-protein linked receptors (GPCR), i.e., AT1 and AT2 receptors; however, in rodents there are two AT1 receptors, the AT1A and AT1B (Chiu et al., 1989). AT1 receptors are coupled to several types of Go subunits (Sasamura et al., 2000), whereas AT2 receptors are predominantly coupled to Goi/o (Zhang and Pratt, 1996; Hansen et al., 2000). Recent studies indicate that AT2 receptors signal via three main pathways: 1) generation of the vasodilator nitric oxide (Carey et al., 2000); 2) activation of PLA2 and subsequent formation of arachidonic acid metabolites (Harwalkar et al., 1998); and 3) activation of phosphoprotein phosphatases (Fischer et al., 1998; Tsuzuki et al., 1996).

Studies in Ang II receptor knockout mice have revealed different physiological roles for AT1 and AT2 receptors in the regulation of the cardiovascular system. AT2 receptor knockout mice have increased blood pressure compared to controls and have enhanced responses to treatment with Ang II (Siragy et al., 1999; Akishita et al., 1999). Conversely, AT1A/AT1B receptor double knockout mice have lower blood pressure compared to controls and lack the typical increase in blood pressure to exogenous Ang II (Oliverio et al., 1998). Additionally, the AT2 receptor acts as a vasodepressor receptor in the hypertensive (mRen-2)27 transgenic rat (Nishioka et al., 1998), and recent studies indicate that AT2 receptors mediate sustained vasorelaxation in Wistar-Kyoto rats (WKY) (Widdop et al., 2002). Furthermore, experiments conducted in rats that are not genetically modified indicate that AT2 receptors indeed counteract the effects of AT1 receptors on vascular contraction and growth (Endo et al., 1998; Carey et al., 2001; Moore et al., 2001; Akishita et al., 2000). Therefore, the AT2 receptor buffers AT1 receptor-mediated effects on vascular smooth muscle cells, and, as demonstrated by studies in knockout mice, changes in the relative activity of the two signaling pathways significantly alter vascular responses to Ang II.
Vascular growth, contraction and generation of superoxide in response to Ang II may be determined in part by activation of phospholipase D (PLD). In vascular smooth muscle, Ang II activates the ERK1/2 pathway (Kubo et al., 1999), which in turn contributes to Ang II-induced growth (Chen et al., 1992) and contraction (Touyz et al., 1999; Muthalif et al., 2000). Recent work from our laboratory has shown that PLD, via the formation of phosphatidic acid, plays a critical role in this signal transduction process due to a specific role of phosphatidic acid in the translocation of Raf-1 to the membrane (reviewed in Andresen et al., 2002). Likewise, phosphatidic acid is involved in generation of superoxide (Palicz et al., 2001), and inhibition of PLD in vascular smooth muscle attenuates Ang II-mediated generation of superoxide (Touyz and Schiffrin, 1999).

Despite the key role of PLD in Ang II-mediated signal transduction processes in vascular smooth muscle cells and the evidence that AT2 receptors buffer the vascular responses mediated by AT1 receptors, there is currently no information on the effects of AT2 receptor signaling on AT1 receptor-induced PLD activity. The fact that the AT2 receptor is expressed primarily during development and wound healing and only sparsely in a few organs of adult animals (Nouet and Nahmias, 2000) explains, at least in part, the lack of attention received by AT2 receptor signaling in the regulation of PLD and other regulatory pathways. Previous studies indicate that Ang II-mediated PLD activity is increased in spontaneously hypertensive rats (SHR) compared to WKY smooth muscle cells, and the AT1 receptor is responsible for activating PLD (Freeman and Tallant, 1994; Andresen et al., 2001). We hypothesized that this difference in responses to Ang II may be due to differences in the levels of receptors on WKY and SHR cells.

In the present study we show that AT2 receptors are expressed by cultured preglomerular vascular smooth muscle cells (PGSMCs) obtained from WKY and SHR. The AT1-to-AT2
receptor ratio was 1:1 in WKY and 3:1 in SHR PGSMCs. These findings provided an ideal model system for investigating the role of endogenous AT₂ receptors in modulating AT₁ receptor-induced activation of PLD.
METHODS

MATERIALS USED:

Angiotensin II, PD-123,319, L-158,809, CGP-42112A and collagenase type IV were obtained from Sigma (St. Louis, MO). [3H]palmitate and ^125^-Sar^1^-Ile^8^-Ang II were obtained from NEN/Perkin Elmer (Boston, MA). The AT1 receptor antibody, sc-1173, was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All lipids were obtained from Avanti Polar Lipids (Alabaster, AL).

CELL CULTURE:

All cell culture reagents were obtained from Invitrogen/GibcoBRL (Carlsbad, CA). Six 13 to 15 week-old SHR and WKY rats from Taconic Farms (Germantown, NY) were used to acquire the PGSMCs as previously reported in detail (Jackson et al., 1997). Briefly, 1% Fe_2O_3 DMEM was forcefully injected into isolated kidneys through the renal artery. The iron-loaded kidney was removed from the rat, the cortex was minced, the microvessels (accurate, interlobular and afferent arteriolar) were obtained using a magnet, and the microvessels were incubated in collagenase type IV (0.6 mg/ml). PGSMVs were obtained by tissue culture and purified by differential plating. Experiments were conducted between passage 1 and 10, and the PGSMCs were grown in DMEM/F12 supplemented with 10% FBS and 1x penicillin/streptomycin/amphotericin B. Phospholipase D activity was measured as previously described (Shome et al., 2000).

QUANTIFICATION OF mRNA:

Total RNA was isolated from confluent 100-mm plates utilizing the Qiagen Rneasy Mini Kit protocol (Valencia, CA), and 10 µl of total RNA was used in the Clontech Advantage RT-
for-PCR Kit (Palo Alto, CA). The resulting cDNA was used to amplify AT₁ receptor, AT₂ receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. GAPDH was used as an internal control in each reaction. The primers for the AT₁ receptors were designed to amplify both AT₁A and AT₁B generating the same size band (Table 1), and the amplification cycles were designed so that all the reactions would occur simultaneously. The products were run on 2% agarose gels subsequently stained with ethidium bromide. Digital images of the gels were analyzed utilizing Molecular Analyst for Windows NT (Molecular Dynamics, Biorad, Hercules, CA).

**Western Blots:**

Confluent PGSMCs were frozen in liquid nitrogen and ground into a powder on liquid nitrogen. Samples were placed in a tube with 0.5 ml lysis buffer (50 mM Tris, pH 7.0, 2% SDS, 10% glycerol, 2 μg/ml antipain, 1 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mg/ml PMSF) and homogenized. The homogenate was centrifuged at 12,000 rpm at 4°C for 10 minutes, and the supernatant was recovered. Protein concentration was determined by the copper bicinchoninic acid method, and samples were stored at -20°C.

**125I-SAR¹-Ile⁸-ANG II BINDING:**

PGSMCs were grown to confluence in 6 well plates, washed, and serum starved overnight. The next day the cells were washed twice for 5 min in 1.5 mL binding buffer (50mM Na₂HPO₄, 150 mM NaCl, 10 mM MgCl₂, and 0.05% bovine serum albumin pH = 7.1) and treated in 1mL binding buffer with 30 pmoles (final concentration, 30 nmoles/L) ¹²⁵I-Sar¹-Ile⁸-Ang II. Various amounts of Ang II, L-158,809 (an AT₁ receptor antagonist) or PD-123,319 (an
AT₂ receptor antagonist) were added at room temperature for 60 min. The PGSMCs were quickly washed with 1 mL binding buffer and lysed in 10% SDS. Protein concentration was determined by the BCA method, and the amount of \(^{125}\text{I-Sar}^1\text{-Ile}^8\text{-Ang II}\) bound was determined by \(\gamma\)-counting. To convert the binding data to number of cells, the number of cells in each sample was determined by counting multiple aliquots of WKY and SHR PGSMCs and determining the total protein in aliquots from the same samples. Thus the following simple formula was experimentally determined to convert the binding data to cell number: \(\mu\text{g protein in sample} \times \text{(# of cells/}\mu\text{g protein)}\). For WKY and SHR PGSMCs, the number of cells per \(\mu\text{g protein}\) was 6064 ± 234 and 9212 ± 370, respectively. This formula, the specific activity of \(^{125}\text{I-Sar}^1\text{-Ile}^8\text{-Ang II}\) and the results from the \(\gamma\)-counter were used to determine the amount of \(^{125}\text{I-Sar}^1\text{-Ile}^8\text{-Ang II}\) bound per cell.

**DATA AND STATISTICAL ANALYSIS:**

For all mathematical operations containing two independent data sets with a measurable error, the following error propagation formulas were applied. If \(f\) and \(g\) are two means and \(f_e\) and \(g_e\) are their respective error, then the error for \(f/g\) is \([f_e x g - f x g_e]/g^2\) and the error for \(f \pm g\) is \(f_e + g_e\). For multiple comparisons, the data were analyzed by ANOVA with Fisher’s LSD 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 using the NCSS 2000 software package (Kaysville, UT), and dose response curves were analyzed using the curve fit routines of GraphPad Prism 4 (San Diego, CA).
RESULTS

DETERMINATION OF ANG II RECEPTOR EXPRESSION.

The amounts of AT₁ and AT₂ receptor mRNA present in PGSMCs was determined by RT-PCR using GAPDH as an internal control (Fig. 1). Both WKY and SHR PGSMCs expressed mRNA for both receptor subtypes. However, SHR PGSMCs expressed approximately 2-fold more AT₁ receptor mRNA compared with WKY PGSMCs. Also, SHR PGSMCs expressed less AT₂ receptor mRNA compared with WKY PGSMCs. Additionally, SHR PGSMCs contained significantly more AT₁ receptor mRNA compared with AT₂ receptor mRNA, whereas WKY PGSMCs contained similar amounts of both types of mRNA (Table 2). To test the validity of these results, Western blots were conducted on WKY and SHR PGSMCs for the AT₁ receptor (Fig. 2). In accordance with the RT-PCR data the SHR PGSMCs expressed approximately 2-fold more AT₁ receptors than WKY PGSMCs. We attempted to perform a similar analysis for the AT₂ receptor; however, due to very high levels of nonspecific binding of the commercially available AT₂ receptor antibodies we were unable to obtain reliable Western blots in this regard.

Because we lacked the Western blot conformation of the RT-PCR studies for the AT₂ receptor and because receptor mRNA does not necessarily correlate with receptor expression, binding experiments with the Ang II antagonist $^{125}$I-Sar¹-Ile⁸-Ang II were conducted to determine the number of Ang II receptors on the cell surface (Fig. 3). Displacement of $^{125}$I-Sar¹-Ile⁸-Ang II with unlabeled Ang II (Fig. 3A) indicated that the expression of Ang II receptors in SHR PGSMCs was marginally greater than that in WKY PGSMCs, 1356 ± 86 versus 897 ± 50 receptors per cell, respectively. To examine the expression of AT₁ versus AT₂ receptors, we used similar $^{125}$I-Sar¹-Ile⁸-Ang II displacement protocols with AT₁ and AT₂ receptor specific antagonists. The data obtained with the AT₁ receptor antagonist L-158,809 (Fig. 3B) indicated
that the level of expression of AT₁ receptors was significantly greater in SHR than in WKY PGSMCs, 692 versus 356 receptors per cell, respectively. In contrast, displacement with the AT₂ receptor specific antagonist PD-123,319 (Fig. 3C) showed that SHR PGSMCs expressed significantly fewer AT₂ receptors than WKY PGSMCs, 248 versus 482 receptors per cell, respectively. Importantly, the combination of the displacement with L-158,809 and PD-123,319 resulted in a total Ang II receptor number per cell of 838 ± 147 and 940 ± 149 for WKY and SHR PGSMCs, respectively. In WKY PGSMCs, displacement with Ang II and the antagonists yielded internally consistent results for the total number of receptors. However, in SHR PGSMCs, the L-158,809 plus PD-123,319 did not displace all of the bound ¹²⁵I-Sar¹-Ile⁸-Ang II, suggesting an additional binding site in SHR PGSMCs. Regardless, as indicated in Table 2, the ratio of the levels of expression of AT₁ and AT₂ receptors was similar to the AT₁ receptor-mRNA to AT₂ receptor-mRNA ratio calculated from the RT-PCR studies. Therefore, we conclude that SHR PGSMCs express significantly more AT₁ receptors than AT₂ receptors, resulting in an increased AT₁-to-AT₂ receptor ratio compared with WKY PGSMCs.

EFFECT OF AT₂ RECEPTORS ON ANG II-MEDIATED PLD ACTIVITY.

We previously reported that Ang II is a more potent activator of PLD in SHR than in WKY PGSMCs (Andresen et al., 2001). In this regard, the concentration-response curves to Ang II were characterized by an EC₅₀ of 3.9 nmoles/L and 47 nmoles/L for SHR and WKY PGSMCs, respectively (see Supplemental Fig.). The increased potency of Ang II-mediated PLD activity in SHR PGSMCs may be due to the greater AT₁-to-AT₂ receptor ratio observed in the SHR cells. Accordingly, in the present study, we examined the role of AT₁ versus AT₂ receptors in mediating Ang II-induced PLD activity.
As shown in Figure 4A, in both WKY and SHR PGSMCs, basal PLD activity was not significantly affected by L-158,809 (an AT\textsubscript{1} receptor antagonist), PD-123,319 (an AT\textsubscript{2} receptor antagonist) or CGP-42112A (an AT\textsubscript{2} receptor agonist). In both WKY and SHR PGSMCs, a high, saturating concentration of Ang II (1 \(\mu\)mole/L) increased PLD activity (Fig. 4B), and this response was blocked by L-158,809, but not by PD-123,319. These data confirm that AT\textsubscript{1} receptors, not AT\textsubscript{2} receptors, mediate Ang II-induced PLD activity.

To examine the hypothesis that the AT\textsubscript{1}-to-AT\textsubscript{2} receptor ratio determines the effects of Ang II on PLD activation, we examined Ang II-induced PLD activity concentration-response curves in the absence and presence of the specific AT\textsubscript{2} receptor antagonist PD-123,319. The rationale for these experiments was that in the presence of the AT\textsubscript{2} receptor antagonist, the differences between SHR and WKY PGSMCs should be significantly reduced by PD-123,319 if the AT\textsubscript{2} receptor importantly modulates the effects of the AT\textsubscript{1} receptor on PLD activity. As shown in Figure 5A, addition of the AT\textsubscript{2} receptor antagonist shifted the Ang II EC\textsubscript{50} of WKY PGSMCs from 63 nmoles/L to 4.2 nmoles/L, a value that is consistent with the EC\textsubscript{50} of 8.8 nmoles/L calculated for the SHR cells (Fig. 5B). The effect of PD-123,319 on SHR cells was not significant (Fig. 5B), a finding consistent with the reduced AT\textsubscript{2} receptor expression in SHR cells.

The hypothesis that AT\textsubscript{2} receptor signaling attenuates Ang II-mediated PLD activation was further examined using the AT\textsubscript{2} receptor agonist CGP-42112A. As with PD-123,319, CGP-42112A alone has no significant effect on PLD activity (Fig. 4A). However, CGP-42112A significantly altered the Ang II-mediated PLD activity concentration-response curves of WKY and SHR PGSMCs (Fig. 5A and 5B). The WKY curve was shifted significantly downwards resulting in a 60% decrease in efficacy (Fig. 5A), whereas the SHR concentration-response curve
was shifted significantly rightward resulting in an EC$_{50}$ = 31 nmoles/L, compared to an EC$_{50}$ of 8.8 nmoles/L in the absence of the agonist (Fig. 5B).
DISCUSSION

The present study demonstrates that cultured WKY and SHR PGSMCs express both AT_1 and AT_2 receptors. Our study also indicates that the ratio of AT_1-to-AT_2 receptors is greater in cultured SHR versus WKY PGSMCs. Several reports suggest that AT_2 receptors attenuate AT_1 receptor-mediated responses. Therefore, the greater potency of Ang II on PLD activity in SHR compared with WKY PGSMCs is consistent with the interpretation that AT_2 receptors attenuate AT_1 signaling. This conclusion is further supported by the observations that an AT_2 receptor antagonist enhances and an AT_2 receptor agonist inhibits Ang II-mediated PLD activation more so in WKY PGSMCs that express a lower AT_1-to-AT_2 receptor ratio compared with SHR PGSMCs. Taken together, our data provide strong evidence that AT_2 receptors attenuate AT_1 receptor-induced PLD activity.

Because an AT_2 receptor antagonist enhances and an AT_2 receptor agonist inhibits Ang II-mediated PLD activation, these studies indicate that the mechanism by which AT_2 receptors inhibit AT_1 receptor-mediated PLD activation involves AT_2 receptor-mediated signaling rather than via heterodimerization and subsequent formation of an inactive AT_1-AT_2 receptor complexes as has been previously suggested (AbdAlla et al., 2001). An important issue is the mechanism by which AT_1 and AT_2 receptors signal to PLD and how those signals interact. In this regard, our previously published studies demonstrate that AT_1 receptors signal to PLD in PGSMCs from both SHR and WKY via a RhoA dependent mechanism (Andresen et al., 2001), however the signal transduction cascade activated by the AT_2 receptor responsible for inhibiting AT_1 receptor-mediated PLD activity remains undefined.

Vascular smooth muscle cells in vivo express high levels of AT_2 receptors during fetal development (Hutchinson et al., 1999) and following vascular injury (Suzuki et al., 2002).
Moreover, AT₂ receptors mediate vasodilation in selected vascular beds (Widdop, et al., 2002). These findings have motivated researchers to examine signaling mechanisms of AT₂ receptors in vascular smooth muscle cells in vitro. However, efforts in this regard have been hampered by the fact that most vascular smooth muscle cells in vitro express low to undetectable levels of native AT₂ receptors (Stoll et al., 1995). Consequently, investigators have relied upon in vitro model systems involving overexpression of AT₂ receptors in vascular smooth muscle cells using various transfection protocols. Unfortunately, this approach has limitations in that overexpression of receptors may lead to artifacts, such as receptor oligomerization (Ramsay et al., 2002) and engagement of illicit signal transduction pathways (Wellner-Kienitz et al., 2001). Our model system is unique in that PGSMCs express both AT₁ and AT₂ receptors without transfection; moreover, the ratio of AT₁-to-AT₂ receptors is dependent on the strain from which the PGSMCs are obtained. Therefore, PGSMCs from SHR and WKY afford an ideal model system for investigating the role and signal transduction pathways of AT₂ receptors in vascular biology.

It should be noted that previous reports did not identify AT₂ receptors in the renal vasculature (Haddad and Garcia, 1996; Song et al., 1995; Song et al., 1994; Gao et al., 2003). Indeed, we have been unable to detect AT₂ receptors in freshly isolated preglomerular microvessels from either WKY or SHR using either Western blots or RT-PCR (Gao et al., 2003). There are at least two explanations for the presence of AT₂ receptors in cultured PGSMCs, but not freshly isolated microvessels. It is possible that the phenotype of PGSMCs changes in vitro such that Ang II receptor expression patterns are altered in culture. However, it is equally possible that PGSMCs that migrate from isolated preglomerular microvessels into a culture dish and survive differential plating are a subset of cells that do not represent the average phenotype.
of the larger population of PGSMCs in the preglomerular vasculature. Thus it is important to note that the inability to detect AT2 receptors in intact preglomerular microvessels does not rule out the expression of AT2 receptors in a subset of PGSMCs in vivo.

Regardless of whether AT2 receptor-expressing PGSMCs are induced or selected, the experiments reported here demonstrate conclusively that AT2 receptors attenuate the ability of Ang II to activate PLD. This conclusion has important implications. Previous studies show that the expression of AT2 receptors is increased in injured arteries (Viswanathan and Saavedra, 1992). Because PLD activity is critical to activation of the ERK cascade, which leads to vascular proliferation, and AT2 receptors inhibit PLD activation, AT2 receptors may play a significant role in the attenuation of the proliferation of vascular smooth muscle cells and the development of vaso-occlusion following vascular injury. Additionally, because PLD is involved in generation of reactive oxygen species, AT2 receptor-mediated inhibition of PLD activity may be partially responsible for the observed AT2 receptor-mediated reduction of the generation of superoxide induced by Ang II (Rueckschloss et al., 2002). Since our studies demonstrate that the genetic background strongly influences the ratio of AT1-to-AT2 receptors, this may explain why some patients experience restenosis following angioplasty while others do not. This may also explain why some patients are genetically susceptible to vaso-occlusive disorders while others are resistant. Finally, our data suggest that WKY and SHR PGSMCs may provide a useful model system for elucidating the genetic mechanisms that regulate the relative expression of AT1 and AT2 receptors.

In conclusion, cultured PGSMC express both AT1 and AT2 receptors. AT1 receptors mediate the activation of PLD by Ang II, whereas AT2 receptors inhibit AT1 receptor signaling to PLD. The differences between SHR and WKY PGSMCs regarding their Ang II-mediated
PLD activity responses are likely a result of an altered ratio of AT₁-to-AT₂ receptors in these two strains. Our work suggests that genetic mechanisms regulate the AT₁-to-AT₂ receptor ratio in vascular smooth muscle cells, and such genetic mechanisms may determine in part the susceptibility of human beings to vaso-occlusive disorders. The WKY and SHR PGSMCs thus provide an excellent model system for analysis of the genetic basis for Ang II receptor levels and for examining the contribution of endogenous AT₂ receptor signaling to the effects of Ang II stimulation.


Touyz RM and Schiffrin EL (1999) Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. Hypertension 34:976-982.


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Figure Legends

Figure 1: Semi-quantitative RT-PCR of AT₁ and AT₂ receptors.

Levels of AT₁ receptor mRNA were higher in SHR (solid bars) compared to WKY (open bars) PGSMCs, and levels of AT₂ receptor mRNA were lower in SHR compared to WKY PGSMCs; however, total levels of angiotensin receptor mRNA were similar between the two strains. SHR had a four-fold greater level of AT₁ receptor mRNA compared to AT₂ receptor mRNA, whereas WKY PGSMCs had similar mRNA levels for AT₁ receptors versus AT₂ receptors. Data are expressed as mean ± SEM, n = 3; * indicates that the SHR is significantly different (P < 0.05) compared to WKY by t-test.

Figure 2: Western blot of AT₁ receptors.

Levels of AT₁ receptor protein are greater in SHR compared with WKY PGSMCs. Data are expressed as mean ± SEM, n = 6; * indicates that the SHR is significantly different (P < 0.001) compared to WKY by t-test.

Figure 3: Estimation of AT₁ receptor and AT₂ receptor number.

(A) Displacement of ¹²⁵I-Sar₁-Ile₈-Ang II with unlabeled Ang II indicates that SHR PGSMCs (■) had slightly more total Ang II binding sites than WKY PGSMCs (□). (B) Displacement of ¹²⁵I-Sar₁-Ile₈-Ang II with L-158,809 indicates that SHR PGSMCs had 2-fold more AT₁ receptors than WKY; inset is the WKY curve shown with a reduced scale and 95% confidence intervals (dashed lines). (C) Displacement with PD-123,319 indicates that WKY PGSMCs had 2-fold more AT₂ receptors than SHR; inset is the SHR curve shown with a reduced
scale and 95% confidence intervals (dashed lines). n = 3 for all experiments and the experiments were conducted in parallel.

Figure 4: Effect of Ang II receptor agonists and antagonists on PLD activity.

(A) Treatment of WKY (open bars) and SHR (solid bars) PGSMCs with 1.5 nmoles/L (5 x IC50) of the AT1 receptor antagonist L-158,809 (L), 15 nmoles/L (5 x IC50) of the AT2 receptor antagonist PD-123,319 (PD) and 100 nmoles/L of the AT2 receptor agonist CGP-42112A (CGP) did not affect basal PLD activity. (B) PLD activity stimulated by a saturating concentration of Ang II (1µmole/L) was significantly attenuated by L-158,809, but not by PD-123,319, in both WKY and SHR PGSMCs. In SHR PGSMCs, PD-123,319 significantly increased Ang II-mediated PLD activity, and in WKY, CGP-42112A tended to reduce Ang II-mediated PLD activity in WKY PGSMCs. Data are expressed as mean ± SEM, n ≥ 3; bars with different letters are significantly different (P < 0.05).

Figure 5: Effect of PD-123,319 and CGP-42112A on Ang II-mediated PLD activation.

PGSMCs were treated with Ang II (black line, squares), Ang II + 15 nmoles/L of the AT2 receptor antagonist PD-123,319 (light grey line, circles) or Ang II + 100 n mole/L of the AT2 receptor agonist CGP-42112A (grey dotted line, diamonds). (A) In WKY PGSMCs, PD-123,319 significantly increased the potency of Ang II with regard to increasing PLD activity, whereas CGP-42112A significantly decreased the efficacy of Ang II-mediated PLD activity. (B) In SHR PGSMCs PD-123,319 had little effect on the Ang II concentration response curve, and CGP-42112A significantly reduced the potency of Ang II with regard to stimulating PLD activity. Data are expressed as mean ± SEM, n ≥ 3. ANOVAs were used to determine significance.
Supplemental Figure: Ang II more potently activates PLD in SHR compared with WKY PGSMCs.

Ang II stimulated PLD activity in WKY (□) and SHR (■) PGSMCs. However, Ang II was a more potent activator of PLD in SHR compared with WKY PGSMCs. Data are expressed as mean ± SEM, n ≥ 3. ANOVA analysis indicates that the curves are significantly different (P < 0.05).
Table 1: Primers used for RT-PCR. The reverse primers were designed to bind to the complement strand of cDNA.

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Table 2: AT<sub>1</sub>-to-AT<sub>2</sub> Receptor Ratios

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Figure 1

Band Intensity (Ang II receptor/GAPDH)

- **AT₁ Receptor**
- **AT₂ Receptor**
- **Total Ang II Receptors**

* indicates significance.
Figure 2

The figure shows a comparison of AT1R density between WKY and SHR. The density is measured in arbitrary units. SHR has a significantly higher density compared to WKY, as indicated by the asterisk (*) on the graph.
Figure 3
Figure 4

A

PtdEtOH (% of total lipid)

0.4
0.3
0.2
0.1
0.0

Basal
L
PD
CGP

B

PLD Activity (Fold over control)

4
3
2
1
0

Control
1 microM Ang II
L
PD
CGP

Control
1 microM Ang II
L
PD
CGP
Figure 5

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log EC_{50}</th>
<th>Log Std.Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II</td>
<td>-7.202</td>
<td>0.3362</td>
</tr>
<tr>
<td>+ PD</td>
<td>-8.373</td>
<td>0.5239</td>
</tr>
<tr>
<td>+ CGP</td>
<td>Not sigmoidal, no EC_{50}</td>
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B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log EC_{50}</th>
<th>Log Std.Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II</td>
<td>-8.056</td>
<td>0.3036</td>
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<tr>
<td>+ PD</td>
<td>-7.625</td>
<td>0.3935</td>
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<tr>
<td>+ CGP</td>
<td>-7.513</td>
<td>0.3614</td>
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