Phospholipase C Responses in Cells From Spontaneously Hypertensive Rats

Tomohiro Osanai and Michael J. Dunn

We tested the hypothesis that increased systemic vascular resistance in spontaneously hypertensive rats may be secondary to enhanced phospholipase C activity in response to vasoconstrictor stimuli. Activation of phospholipase C by angiotensin II (Ang II), thromboxane A₂, arginine vasopressin, and endothelin-1 was compared in cultured glomerular mesangial cells and mesenteric vascular smooth muscle cells taken from 13- to 14-week-old hypertensive and normotensive Wistar-Kyoto rats (blood pressure, 185±1 versus 135±2 mm Hg). Phospholipase C was assessed by measuring cytosolic free calcium and by the accumulation of radiolabeled inositol phosphates. Basal cytosolic calcium did not differ between mesangial cells taken from both strains but was greater in smooth muscle cells from hypertensive rats (210.1±8.2 versus 149.2±4.7 nM). The responsiveness of cytosolic calcium and inositol phosphate accumulation to Ang II was significantly enhanced in mesangial cells from hypertensive rats (10⁻⁷ M Ang II: peak increase of calcium, 1,266±181 versus 603±93 nM; percent increment of inositol phosphates at 1 minute, 266±26 versus 98±10%). Vascular smooth muscle cells from hypertensive rats, when compared with normotensive rats, showed a similar augmentation of Ang II–stimulated intracellular calcium and inositol phosphates. Thromboxane A₂–induced enhancement of intracellular calcium and inositol phosphate accumulation in vascular smooth muscle cells was also greater in hypertensive animals. However, the responses to vasopressin and endothelin in mesangial or vascular smooth muscle cells did not differ between the normotensive and hypertensive animals. There was no significant difference in Ang II receptor number and affinity between hypertensive- and normotensive-derived mesangial cells. We conclude that genetically increased blood pressure in rats may be secondary to enhanced post-receptor signaling in glomerular mesangial cells activated by Ang II and to enhanced signaling in vascular smooth muscle cells stimulated by either Ang II or thromboxane A₂. (Hypertension 1992;19:446–455)

Key Words • calcium • inositol phosphates • vascular smooth muscle • mesangial cells • spontaneously hypertensive rats

Phospholipase C (PLC) is a plasma membrane enzyme that hydrolyzes phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] thereby releasing inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] to the cytosol, and diacylglycerol within the plane of the membrane. The primary function of Ins(1,4,5)P₃ is to mobilize Ca²⁺ from intracellular stores, whereas diacylglycerol is required for the physiological activation of protein kinase C. Increased intracellular free Ca²⁺ ([Ca²⁺]₀) elicits rapid contraction of smooth muscle and nonmuscle cells by myosin light-chain phosphorylation. Activation of protein kinase C initiates slow but sustained vasoconstriction.¹ ²

In the field of hypertension research, early observations of elevated [Ca²⁺]₀, or a rapid turnover rate of phosphoinositides in spontaneously hypertensive rat (SHR)–derived platelets, lymphocytes, and red blood cells,³ stimulated interest in vascular PLC activity, which may be more important in the regulation of arterial blood pressure. Enhanced PLC activity⁴ and elevated [Ca²⁺]₀ have been found under nonstimulated conditions in cultured aortic vascular smooth muscle cells (VSMCs) obtained from SHR. Because various vasoconstrictor agonists exert their effect in arterial VSMCs via an activation of PLC, receptor-mediated responsiveness of vascular PLC activity has also been investigated. One study⁶ has shown that arginine vasopressin (AVP) evokes a greater increase in [Ca²⁺]₀ in SHR than in Wistar-Kyoto (WKY) primary-cultured aortic VSMCs. Other evidence⁷–¹³ has indicated that the responsiveness of PLC activity to angiotensin II (Ang II) or epidermal growth factor is enhanced in SHR–derived aortic VSMCs. Thus, many experiments have pointed to alterations of PLC activity in aortic VSMCs and circulating cells, but none of these tissues or cells regulate vascular resistance and blood pressure. VSMCs from arterioles regulate the systemic vascular resistance, and the contraction of glomerular mesangial cells reduces glomerular filtration rate and ultrafiltration coefficient.¹⁴ Since it still remains uncertain whether abnormalities observed in cells derived from large vessels and circulating blood cells are present in resistance vessels or glomerular mesangial cells, we tested the hypothesis that increased systemic vascular

From the Department of Medicine (T.O., M.J.D.) and the Department of Physiology and Biophysics (M.J.D.), Case Western Reserve University School of Medicine, and Division of Nephrology, University Hospitals of Cleveland, Ohio.

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Address for correspondence: Michael J. Dunn, MD, Department of Medicine, University Hospitals of Cleveland, 2074 Abington Road, Cleveland, OH 44106.

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resistance and decreased glomerular filtration rate (GFR) in SHR may be secondary to enhanced PLC signaling of mesenteric resistance VSMCs and glomerular mesangial cells in response to vasoconstrictor stimul. We report here that PLC signaling that is activated by Ang II is enhanced in mesenteric VSMCs and glomerular mesangial cells from SHR by post-receptor mechanisms.

Methods

Materials

Ten SHR and 10 WKY rats (male, aged 13-14 weeks) were obtained from Charles River, Boston, Mass. Cell culture media were from Hazleton Biologicals, Lenexa, Kan.; fetal bovine serum (FBS) and bovine calf serum (BCS) were purchased from Hyclone, Logan, Utah. Insulin, transferrin, and selenium were obtained from Collaborative Research, Bedford, Mass. Fura-2 (fura-2 acetoxyethylester) was from Molecular Probes, Eugene, Ore. Aclar coverslips were from Allied Engineered Plastics. Myo-[2-3H(N)]inositol, 125I-Ang II, and [3H]PtdIns(4,5)P₂ were obtained from New England Nuclear, Boston, Mass. Anion exchange resin was from Bio-Rad, Richmond, Calif. The thromboxane A₂ (TXA₂) mimetic U46619 was from Upjohn, Kalamazoo, Mich.; this was stored as 10-mM stock solutions in absolute ethanol at -80°C under argon. Endothelin-1 (ET-1), ionomycin, and nifedipine were purchased from Calbiochem Corp., La Jolla, Calif., whereas Ang II, AVP, and all other reagents were of the finest grade available from Sigma Chemical Co., St. Louis, Mo.

Cell Culture

Mesangial cells and mesenteric VSMCs were obtained from 10 ether-anesthetized male 13- to 14-week-old SHRs and 10 age-matched WKY rats. They were studied as five pairs of cells. Rat mesangial cells were grown from glomerular explants using a slightly modified method. The mesenteric arteries and their small uli. We report here that PLC signaling that is activated by Ang II is enhanced in mesenteric VSMCs and glomerular mesangial cells from SHR by post-receptor mechanisms.

Measurement of [Ca²⁺].

Intracellular [Ca²⁺], was determined with the Ca²⁺-sensitive dye fura-2 after the cells were incubated for 24 hours in serum-free media. As reported, confluient mesangial cell monolayers on plastic Aclar coverslips were loaded with 1 μM fura-2 in RPMI-1640 for 40 minutes at 37°C, then washed twice and incubated again for 20 minutes in RPMI, free of fura-2, to allow for intracellular dye cleavage. Confluient mesenteric VSMC monolayers were loaded with 5 μM fura-2 in serum-free DMEM for 20 minutes at 37°C. The coverslips were mounted in a quartz cuvette with 2 ml Krebs-Henseleit HEPES (KHH), pH 7.4, maintained at 37°C with constant stirring. [Ca²⁺], was determined by measuring fluorescence with a University of Pennsylvania Biomedical Instruments Group spectrophotometer at 340 and 380 nm excitation and 510 nm emission. Fluorescence measurements (F) were converted to Ca²⁺ concentrations by determining maximal fluorescence (Fₘₐₓ) with 25-50 μM ionomycin followed by minimal fluorescence (Fₐ₃) with 7.5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 60 mM Tris, pH 10.5. The following formula was then used:

\[ [\text{Ca}^{2+}] = K_D (F - F_{\text{min}})/F_{\text{max}} - F \]

K_D for fura-2 is 224 nM. Autofluorescence by the cells or agonists was negligible. To study the alterations in [Ca²⁺], responsiveness to the vasoconstrictor agonists in SHR-derived mesangial cells and mesenteric VSMCs,
the dose-dependent [Ca\(^{2+}\)], responses to Ang II, AVP, ET-1, and U46619 were compared between SHR and WKY rats. In addition, the mechanism for the increase in [Ca\(^{2+}\)], in response to 10\(^{-7}\) M Ang II was investigated in mesangial cells taken from SHR and WKY rats, using Ca\(^{2+}\)-free KHH, Ca\(^{2+}\)-free KHH with 4 mM EGTA, 30 \(\mu\)M nifedipine, and high potassium (30 mM).

**Measurement of Inositol Phosphates**

Inositol phosphate was measured after myo-[\(^{3}\)H]inositol incorporation into confluent monolayers of rat mesangial cells and mesenteric VSMCs, followed by perchlorate extraction and separation by anion exchange chromatography.\(^{20}\) Cells grown in 12-well dishes (approximately 20,000 cells per well) were incubated for 40–44 hours with 5 \(\mu\)Ci/ml myo-[\(^{2}\)H(N)]inositol in serum-free RPMI-1640 without inositol. The medium was then removed, and the monolayers were washed three times with PBS, pH 7.4, and incubated in KHH at 37°C supplemented with 10 mM LiCl and vasoconstrictor agonists in various concentrations. Incubations were terminated by the addition of 120 \(\mu\)l of 3.3N perchloric acid (PCA), and the dishes were immediately placed on ice for 15 minutes. The monolayers were scraped off with a rubber policeman and transferred to plastic microcentrifuge tubes; the extracts were pooled with an additional rinse of the wells with 480 \(\mu\)l of 0.55N PCA and were centrifuged for 5 minutes at 10,000g. One ml of the supernate was neutralized with 55 \(\mu\)l of 10N KOH, and after 15 minutes on ice, the precipitated PCA extract was pelleted by centrifugation. One-milliliter aliquots of the neutralized supernates were diluted with 9 ml of 5 mM sodium tetraborate and applied onto prewashed 0.8-ml columns packed with AG1X8 (200-400 mesh) formate anion exchange resin. The columns were rinsed twice with 12 ml of a 60 mM sodium formate–5 mM tetraborate solution, and the inositol phosphates were eluted sequentially with 0.2, 0.4, and 1 M NH\(_4\) formate in 0.1 M formic acid for inositol monophosphate (InsP), inositol bisphosphate (InsP\(_2\)), and inositol trisphosphate (InsP\(_3\)), respectively. This technique does not allow for separation of Ins(1,4,5)P\(_3\).

**Figure 1.** Photomicrographs show immunocytochemical staining of \(\alpha\)-smooth muscle cell actin. Panel A: Fine staining of \(\alpha\)-smooth muscle cell actin. Panel B: Negative control in mesenteric vascular smooth muscle cells with omission of anti-\(\alpha\)-smooth muscle cell actin.
and inositol 1,3,4-trisphosphate [Ins(1,3,4)P3]. Each eluate was collected directly in plastic scintillation vials, evaporated to dryness by vacuum centrifugation in a Savant Speed-Vac 200 apparatus, redissolved in 1 ml distilled water, and mixed with 9 ml Formula 963 counting fluid (New England Nuclear) for liquid scintillation counting.

125I-Angiotensin II Binding Assays

125I-Ang II binding assays were carried out by using membranes from mesangial cells. Confluent monolayers in 100-mm Petri dishes were washed twice with HBSS at 4°C. All subsequent procedures were performed at 4°C. Each monolayer was then scraped into 25 ml HBSS. Suspended cells were centrifuged twice at 500g for 5 minutes with an intermediate resuspension in 25 ml HBSS and then homogenized in 2 ml of ice-cold Tris-sucrose buffer (270 mM sucrose buffered with 50 mM Tris-HCl, pH 7.4) using a Teflon-glass homogenizer (five passes at 1,000 rpm). The homogenate was centrifuged in 15 ml Tris-sucrose buffer at 500g for 10 minutes, and the pellet was discarded. The supernate was centrifuged at 40,000g for 15 minutes, and the pellet was washed twice more at 40,000g, once in 50 mM Tris-HCl buffer (pH 7.7) containing 5.0 mM EDTA, and once in EDTA-free Tris-HCl. The resulting pellets were stored at —80°C. The protein content of the membrane fractions was measured spectrophotometrically. The standard assay mixture contained 25 µl 125I-Ang II (0.25–0.5 nM), 100 µl 50 mM Tris-HCl, pH 7.4 (120 mM NaCl, 0.1 mM CaCl2, 9 mM sodium cholate, 50 mM Tris-HCl, pH 7.4) with unlabeled Ang II (10–10 to 10–6 M) and 125 µl membrane preparation (25–50 µg) in 16×100-mm glass tubes at room temperature. After incubating for 60 minutes, bound and free radioligand were separated by rapidly diluting a 250 µl aliquot of the incubation mixture in 2.0 ml of 50 mM Tris-HCl, pH 7.4, supplemented with 120 mM NaCl and 5 mM MgCl2 (wash buffer), at 4°C and then immediately filtering through presoaked Whatman GF/B glass-fiber filters. Each filter was rapidly washed with 20 ml ice-cold wash buffer and counted in a gamma counter. Nonspecific binding was assessed by incubating the membranes in the presence of 10–8 M unlabeled Ang II and was found to be 28.3±3.6% of total binding in WKY rats and 23.4±4.1% in SHR. Specific binding was then determined by subtracting nonspecific from total binding. Competitive displacement studies were analyzed as described by Cheng and Prusoff.21

Plasma Membrane Phospholipase C Assays

Membrane PLC activity of mesangial cells was measured using exogenous PtdIns(4,5)P2.22 The following components were added to the PLC assay: 50 mM HEPES, pH 7.0, 0.1 mM CaCl2, 9 mM sodium cholate, 0.5% (w/v) BSA, 0.004% bacitracin) with unlabeled Ang II (10–10 to 10–6 M) and 125 µl membrane preparation (25–50 µg) in a final volume of 200 µl. The reaction mixture was incubated for 20 minutes at 37°C, and the reaction was stopped with 1 ml chloroform/methanol/concentrated HCl containing 5 mM EGTA. After extraction, a 400 µl portion of the aqueous phase was removed for liquid scintillation counting. Under these assay conditions, the PLC activities were linear with respect to time and protein concentration.

Statistics

Results are presented as mean±SEM. Comparisons were made using two-way analysis of variance (ANOVA) and unpaired t-test.

Results

Blood Pressure

Systolic blood pressures were measured by the tail-cuff method before the rats were killed, and the values were 135±2 mm Hg for WKY rats (n=10) and 185±1 mm Hg for SHR (n=10) (p<0.001).

Figure 2. Representative tracings or waveforms show increments of intracellular calcium concentration ([Ca2+]i) evoked by angiotensin II (Ang II) in mesangial cells (tracings A–D, spontaneously hypertensive rats; tracings A’–D’, Wistar-Kyoto rats). High concentrations of Ang II caused a biphasic increase in [Ca2+]i, which consisted of a spike increase followed by a sustained increase that was greater in spontaneously hypertensive rats than in Wistar-Kyoto rats.
**Ca**^2+^ Studies

**Basal [Ca**^2+^]** did not differ between cultured mesangial cells taken from the hypertensive (161.9±6.2 nM) and normotensive strains (170.8±8.9 nM) but was greater in mesenteric VSMCs taken from SHR (210.1±8.2 nM versus 140.2±4.7 nM, p<0.001). Figures 2, 3, and 4 show the representative tracings or waveforms of [Ca**^2+^]**, evoked by Ang II in mesangial cells and by Ang II and U46619 in mesenteric VSMCs. In mesangial cells (Figure 2), 10^{-7} and 10^{-6} M Ang II caused a biphasic increase in [Ca**^2+^]**, that consisted of a rapid transient or spike increase followed by a smaller but sustained increase. Peak [Ca**^2+^]** was greater in SHR than in WKY rats, and the declination of the sustained phase was steeper in SHR (shown as A,B) compared with WKY rats (shown as A',B'). Ang II (10^{-6} and 10^{-10} M) induced a monophasic increase in [Ca**^2+^]**. In mesenteric VSMCs (Figures 3 and 4), Ang II (10^{-7} and 10^{-6}) and U46619 (10^{-6} and 10^{-7} M in SHR, 10^{-6} M in WKY rats) caused a biphasic increase in [Ca**^2+^]**. The response of [Ca**^2+^]** was greater in SHR than in WKY rats, but there was no difference in the rates of decline of [Ca**^2+^]** between the two strains in VSMCs. The lower concentrations of Ang II and U46619 induced a monophasic sustained increase in [Ca**^2+^]**. Characteristically, the sustained increase induced by U46619 lasted longer than that induced by the other three agonists. Figure 5 shows the dose-dependency of Ang II–, AVP–, U46619–, and ET-1–induced Ca**^2+^** signaling in mesangial cells and mesenteric VSMCs. The dose-dependent peak [Ca**^2+^]** (calculated by subtracting basal from peak [Ca**^2+^]**) in response to Ang II was greater in mesangial cells and VSMCs from SHR than in those from WKY rats (two-way ANOVA: mesangial cells, p<0.05; mesenteric VSMCs, p<0.05). The dose-dependent enhancement of [Ca**^2+^]** in response to TXA2 was greater in VSMCs from SHR than in WKY rats (two-way ANOVA, p<0.05). In contrast, there was no significant difference of peak [Ca**^2+^]** in response to AVP and ET-1 between SHR and WKY mesangial cells. [Ca**^2+^]** increments in response to U46619 in mesangial cells and in response to ET-1 in mesenteric VSMCs were insignificant even when monolayers were exposed to high concentrations of agonists, indicating the loss of these receptors in our primary cultures. The thresholds for the increase in [Ca**^2+^]**, to Ang II, AVP, U46619, and ET-1 did not differ between SHR and WKY strains. The decline of [Ca**^2+^]**, from the peak [Ca**^2+^]**, in mesangial cells stimulated with 10^{-7} M Ang II, AVP, and ET-1 was more rapid in SHR-derived mesangial cells than in WKY cells. However, there were no differences in the [Ca**^2+^]**, decay in VSMCs taken from both strains. When extracellular sodium was replaced by choline, thereby eliminating calcium-sodium exchange, the increments of [Ca**^2+^]** were greater in Ang II–stimulated SHR mesangial cells than in cells from WKY rats.

**Characterization of the Increase in [Ca**^2+^]**

When Ang II was added in nominally Ca**^2+^**-free KHH or in Ca**^2+^**-free KHH after addition of EGTA, the spike phase of [Ca**^2+^]** was attenuated and the sustained phase was abolished in mesangial and VSMCs (data not shown). Nifedipine failed to block either the transient spike or the sustained phase of the calcium response.
or sustained phase of [Ca\(^{2+}\)]\(_i\), in response to Ang II. High extracellular potassium concentration ([K\(^+\)]\(_o\)) failed to increase [Ca\(^{2+}\)]\(_i\) in mesangial and mesenteric VSMCs. These results demonstrate that Ang II-stimulated Ca\(^{2+}\) influx depends on nifedipine-insensitive and voltage-independent Ca\(^{2+}\) channels in these primary cultures.

**Inositol Phosphate Studies**

Initially, we investigated the temporal pattern of InsP, InsP\(_2\), and InsP\(_3\) formation in response to 10\(^{-7}\) M Ang II in SHR and WKY rats. As shown in Figure 6, in mesangial cells a slow increase in InsP accumulation started at 1 minute and plateaued between 5 and 15 minutes. InsP release was 20–30% greater in SHR cells at 15 seconds and 1 minute, but the entire time course did not differ between SHR and WKY rats by two-way ANOVA. In contrast to the temporal pattern of InsP, Ang II at 10\(^{-7}\) M elicited a rapid accumulation of InsP\(_2\) and InsP\(_3\) within 15 seconds. These rapid rises of InsP\(_2\) and InsP\(_3\) reached a maximum at 1 minute after the exposure to Ang II and were followed by a rapid decline toward the basal level. The time course of accumulation of InsP\(_2\) and InsP\(_3\) in mesangial cells was significantly greater in SHR than in WKY rats (two-way ANOVA: InsP\(_2\) and InsP\(_3\), \(p<0.05\)). In mesenteric VSMCs, as illustrated in Figure 7, an increase in InsP accumulation in response to 10\(^{-7}\) Ang II occurred at 1 minute and plateaued between 5 and 15 minutes. Time-dependent InsP accumulation was greater in SHR (two-way ANOVA, \(p<0.05\)). Ang II elicited a rapid accumulation of InsP\(_2\) and InsP\(_3\) within 15 seconds of the application of Ang II, but in contrast to mesangial cells, these increments of InsP\(_2\) and InsP\(_3\) persisted between 1 and 15 minutes. The time-dependent accumulation of InsP\(_2\) and InsP\(_3\) was significantly greater in SHR than in WKY VSMCs (two-way ANOVA: InsP\(_2\) and InsP\(_3\), \(p<0.05\)). Based on these experiments, 1 minute of Ang II exposure in mesangial cells and 5 minutes' exposure in mesenteric VSMCs were judged to be appropriate for the estimation of dose-dependent changes in all inositol phos-

**FIGURE 5.** Line plots show dose-dependency of angiotensin II (Ang II), arginine vasopressin (AVP), U46619, and endothelin-1 (ET-1)-induced changes of intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) in mesangial cells and mesenteric vascular smooth muscle cells (VSMCs). Data are mean±SEM for five to seven monolayers in different experiments.

**FIGURE 6.** Line plots show time-course of inositol mono-, bis-, and trisphosphate (IP\(_1\), IP\(_2\), and IP\(_3\)) formation in response to 10\(^{-7}\) M angiotensin II (Ang II) in mesangial cells. These cells were fifth or sixth passage. Data are mean±SEM for three experiments in duplicate. Basal counts per minute±SEM per well: IP\(_1\), 384±59 spontaneously hypertensive rats (SHR), 342±36 Wistar-Kyoto (WKY) rats; IP\(_2\), 62±4 SHR, 90±17 WKY rats; IP\(_3\), 52±14 SHR, 55±16 WKY rats.
Figure 7. Line plots show time-course of inositol mono-, bis-, and trisphosphate (IP\textsubscript{1}, IP\textsubscript{2}, and IP\textsubscript{3}) formation in response to 10\textsuperscript{-7} M angiotensin II (Ang II) in mesenteric vascular smooth muscle cells (VSMCs). All experiments used third passage cells. Data are mean±SEM for four experiments in duplicate. Basal counts per minute±SEM per well: IP\textsubscript{1} 1,208±267 spontaneously hypertensive rats (SHR), 1,253±226 Wistar-Kyoto (WKY) rats; IP\textsubscript{2} 288±60 SHR, 307±45 WKY rats; IP\textsubscript{3} 184±32 SHR, 184±47 WKY rats.

Figure 8. Line plots show dose-dependent increments of inositol monophosphate (IP\textsubscript{1}), inositol bisphosphate (IP\textsubscript{2}), and total inositol phosphates (IP\textsubscript{3}) stimulated with various concentrations of angiotensin II (Ang II) in mesangial cells. These experiments used fourth and fifth passage cells. Data are mean±SEM for three experiments in duplicate. Basal counts per minute±SEM per well: IP\textsubscript{1} 390±132 spontaneously hypertensive rats (SHR), 317±71 Wistar-Kyoto (WKY) rats; IP\textsubscript{2} 283±205 SHR, 265±115 WKY; IP\textsubscript{3} 77±38 SHR, 53±19 WKY rats.

Discussion
Several publications\textsuperscript{8-12} have documented enhanced PLC activity in VSMCs derived from large conduit vessels, i.e., the aorta. We questioned whether similar changes are present in resistance VSMCs and glomerular mesangial cells, which are modified vascular peri-
Figure 9. Line plots show dose-dependent increments of inositol monophosphate (IP), inositol bisphosphate (IP2), and total inositol phosphates (IPs) stimulated with various concentrations of angiotensin II (Ang II) in mesenteric vascular smooth muscle cells (VSMCs). All experiments used second passage cells. Data are mean±SEM for three experiments in duplicate. Basal counts per minute±SEM per well: IP, 1,768±902 spontaneously hypertensive rats (SHR), 820±185 Wistar-Kyoto (WKY) rats; IP2, 510±286 SHR, 313±143 WKY rats; IP3, 49±23 SHR, 55±16 WKY rats.

Figure 10. Line plots show dose-dependent increments of all inositol phosphate (IPs) fractions stimulated with various concentrations of U46619 in mesenteric vascular smooth muscle cells (VSMCs). Data are mean±SEM for three experiments in duplicate. Basal counts per minute±SEM per well: inositol monophosphate (IP), 1,397±256 spontaneously hypertensive rats (SHR), 953±114 Wistar-Kyoto (WKY) rats; inositol bisphosphate (IP2), 316±35 SHR, 216±1 WKY rats; IP3, 98±60 SHR, 108±69 WKY rats.

as well as larger vessels possess the enhanced PLC signaling by Ang II. Only one study failed to demonstrate an increased [Ca2+]i response to Ang II but did show greater [Ca2+]i increments to AVP.10 However, no data are consistent with our hypothesis.

It is of interest that the hyperresponsiveness of PLC was agonist-specific. With regard to Ang II, previous evidence has shown an increased Ca2+ response in cultured aortic SHR VSMCs that was assessed by 45Ca2+ uptake,24 increased Ang II-stimulated inositol phosphate accumulation,11,12 and enhanced intracellular pH in aortic VSMCs from adult SHR.12,25 Our results from the Ca2+ and inositol phosphate studies are in accord with these reports and show that resistance vasculature

TABLE 1. Inositol Phosphates (Percent Above Control)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>InsP</th>
<th>InsP2</th>
<th>InsP3</th>
<th>Total InsPs</th>
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<tr>
<td>Mesangial cells (1-minute exposure)</td>
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<tr>
<td>ET-1 (10^-7)</td>
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<tr>
<td>SHR</td>
<td>73.5±38.3</td>
<td>313.2±184.7</td>
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<td>55.0±23.8</td>
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<td>34.5±11.4</td>
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<tr>
<td>AVP (10^-7)</td>
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</tr>
<tr>
<td>SHR</td>
<td>78.8±19.8</td>
<td>106.8±45.1</td>
<td>19.8±11.7</td>
<td>76.3±25.8</td>
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<tr>
<td>WKY</td>
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<td>3.3±6.9</td>
<td>23.0±11.9</td>
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<td>Mesenteric VSMCs (5-minute exposure)</td>
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<tr>
<td>AVP (10^-7)</td>
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<tr>
<td>SHR</td>
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<td>292.5±64.9</td>
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<td>WKY</td>
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<td>207.5±70.5</td>
<td>79.7±6.3</td>
<td>221.5±68.5</td>
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</table>

Data are mean±SEM for six (mesangial cells) or three (vascular smooth muscle cells [VSMCs]) experiments in duplicate. Basal counts per minute±SEM per well: mesangial cells, inositol monophosphate (InsP) 531±39 spontaneously hypertensive rats (SHR), 421±80 Wistar-Kyoto (WKY) rats; inositol bisphosphate (InsP2) 456±169 SHR, 312±114 WKY rats; inositol trisphosphate (InsP3) 125±31 SHR, 95±25 WKY rats; VSMCs, InsP 1,768±902 SHR, 820±185 WKY rats; InsP2, 510±286 SHR, 313±143 WKY rats; InsP3, 49±23 SHR, 55±16 WKY rats. ET-1, endothelin-1; AVP, arginine vasopressin.
The Ang II-induced increases of inositol phosphates in SHR mesangial cells and VSMCs, compared with WKY cells, appeared at lower threshold concentrations of Ang II (Figures 8 and 9) than the enhanced responsiveness of [Ca\(^{2+}\)] in SHR mesangial cells and VSMCs, compared with WKY rats. This discrepancy may be due to the difference of study design: suspended aortic VSMCs from young (5-week-old) SHR were used to measure [Ca\(^{2+}\)], with quin-2. In contrast to the well-studied Ang II-induced signaling in VSMCs, nothing has been reported about PLC signaling of SHR mesangial cells activated by Ang II or TXA\(_2\)-stimulated PLC signaling of VSMCs from SHR. In the present study we provided evidence that the increased PLC response to Ang II exists in SHR-derived mesangial cells and that PLC of mesenteric VSMCs from SHR are hyperresponsive to TXA\(_2\) as well as to Ang II. Because vascular TXA\(_2\) production is increased in SHR,\(^{26}\) the TXA\(_2\)-induced enhancement of PLC and [Ca\(^{2+}\)], in SHR VSMCs might play an important role in arterial hypertension.

As demonstrated in coarctation-induced hypertension,\(^{27}\) elevation of blood pressure in SHR may cause secondary enhancement of inositol phosphate formation. However, since increased PLC activity and increased [Ca\(^{2+}\)], are already observed in prehypertensive SHR\(^{8,28}\) and since these abnormalities are not restricted to contractile vascular tissues,\(^{5-7,29}\) alterations of PLC in SHR appear to be a primary genetic defect. In recent years persuasive evidence has been advanced showing that the properties of guanine nucleotide-binding proteins (G proteins)\(^{30-35}\) and PLC\(^{36-38}\) differ among various types of cells and kinds of receptors. Some studies have demonstrated that abnormalities of G protein or PLC may be present in this genetic hypertensive model.\(^{39}\) Enhancement of Ang II–induced PLC and [Ca\(^{2+}\)], responses, without changes of ET-1 and AVP-stimulated PLC and [Ca\(^{2+}\)], may be secondary to a specific, genetic alteration of the G protein linking the Ang II receptor to PLC. It is unknown whether different isoforms of G proteins associate diverse receptors to PLC. It is also possible that the genetic defect leading to hypertension in SHR is in the PLC linked to specific receptors (i.e., Ang II). Our experiments with mesangial cell plasma membranes as well as previously published data support the belief that PLC enzymatic activity is greater in cells from SHR than in those from WKY rats. We do not know whether there are differences of PLC isotypes between these rat species. In addition to G protein and PLC itself, the PLC regulatory proteins\(^{40,41}\) and the amount of PtdIns(4,5)P\(_2\) in the plasma membrane could also affect the activity of PLC. However, these explanations fail to explain the specificity of agonist-induced PLC hyperresponsiveness.

The Ang II–induced increases of inositol phosphates in SHR mesangial cells and VSMCs, compared with WKY cells, appeared at lower threshold concentrations of Ang II (Figures 8 and 9) than the enhanced responsiveness of [Ca\(^{2+}\)] to Ang II (Figure 5). It should be emphasized that we measured mesangial inositol phosphates at 1 minute, VSMC inositol phosphates at 5 minutes, and peak [Ca\(^{2+}\)], at variable times ranging from 15 to 30 seconds (high concentrations of Ang II) to 1–2 minutes (Ang II 0.1–1.0 nM). In addition, SHR cells may have an augmented calcium efflux across the plasma membrane, thereby attenuating the PLC-dependent increments of [Ca\(^{2+}\)], in response to Ang II (T. Osanai and M.J. Dunn, unpublished observations).

Enhanced responsiveness of VSMCs and glomerular mesangial cells to vasoconstrictor stimuli may increase blood pressure acutely and chronically. Acutely, Ang II may cause greater contraction of resistance arterioles, thereby altering total peripheral resistance. Chronically, greater proliferation of SHR VSMCs in response to Ang II would lead to medial hyperplasia and hypertrophy thereby reducing arteriolar lumenal diameter. In addition, mesangial contraction reduces glomerular filtration rate through decrements of glomerular filtration surface area thereby possibly augmenting positive sodium and volume balances.

We conclude that increased blood pressure in SHR may be secondary to enhanced post-receptor signaling of glomerular mesangial cells activated by Ang II and of VSMCs activated by Ang II and TXA\(_2\).

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**Table 2. Mesangial Cell Angiotensin II Receptor Number and Affinities**

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>B(_{\text{max}}) (fmol/mg protein)</th>
<th>K(_{d}) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR (n=4)</td>
<td>303±62</td>
<td>0.67±0.11</td>
</tr>
<tr>
<td>WKY (n=4)</td>
<td>263±45</td>
<td>0.63±0.08</td>
</tr>
</tbody>
</table>

Data are mean±SEM for four experiments in duplicate. B\(_{\text{max}}\), maximum binding; K\(_{d}\), 50% displacement value.

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**Figure 11.** Line plot shows competitive displacement analysis of \(^{125}\)I-Angiotensin II (Ang II) binding to mesangial cell membrane. Each point represents specific \(^{125}\)I-Ang II binding (%B–NS/B\(_{-}\)–NS). B\(_{\text{max}}\), total binding; B, binding in presence of various concentrations of unlabeled Ang II; NS, nonspecific binding; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats. Data are mean±SEM for four experiments in duplicate.
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