Differential $[\text{Ca}^{2+}]_i$ Signaling of Vasoconstriction in Mesenteric Microvessels of Normal and Reduced Uterine Perfusion Pregnant Rats

Wensheng Chen and Raouf A. Khalil

Division of Vascular Surgery,
Brigham and Women’s Hospital,
and Harvard Medical School, Boston, MA

Running Title: Microvessel $\text{Ca}^{2+}$ Sensitivity during Pregnancy

Correspondence and Reprints:
Raouf A Khalil, MD, PhD
Harvard Medical School
Brigham and Women’s Hospital
Division of Vascular Surgery
75 Francis Street
Boston, MA 02115
Tel: (617) 525-8530
Fax: (617) 264-5124
E-mail: raouf_khalil@hms.harvard.edu

List of Abbreviations:
AngII, angiotensin II; BP, blood pressure; $[\text{Ca}^{2+}]_i$, intracellular free $\text{Ca}^{2+}$ concentration; ET-1, endothelin-1; HTN-Preg, hypertension in pregnancy; Norm-Preg, normal pregnant; Phe, phenylephrine; PKC, Protein Kinase C; RUPP, Reduced Uterine Perfusion Pressure; VSM, vascular smooth muscle
ABSTRACT

Vascular resistance and blood pressure (BP) are reduced during late normal pregnancy (Norm-Preg). In contrast, studies in human preeclampsia and in animal models of hypertension in pregnancy (HTN-Preg) have suggested that localized reduction in uterine perfusion pressure (RUPP) in late pregnancy is associated with increased systemic vascular resistance and BP; however, the vascular mechanisms involved are unclear. Because Ca\(^{2+}\) is a major determinant of vascular contraction, we hypothesized that the [Ca\(^{2+}\)]\(_i\) signaling of vasoconstriction is differentially regulated in systemic microvessels during normal and RUPP in late pregnancy. Pressurized mesenteric microvessels from Norm-Preg and RUPP rats were loaded with fura-2 in preparation for simultaneous measurement of diameter and [Ca\(^{2+}\)]\(_i\) (presented as fura-2 340/380 ratio). Basal [Ca\(^{2+}\)]\(_i\) was lower in RUPP (0.73±0.03) compared to Norm-Preg rats (0.82±0.03). Membrane depolarization by 96 mM KCl, phenylephrine (Phe, 10\(^{-5}\) M), angiotensin II (AngII, 10\(^{-7}\) M), or endothelin-1 (ET-1, 10\(^{-7}\) M) caused an initial peak followed by maintained vasoconstriction and [Ca\(^{2+}\)]\(_i\). KCl caused similar peak vasoconstriction and [Ca\(^{2+}\)]\(_i\) in Norm-Preg (45.5±3.3%, 0.89±0.02) and RUPP rats (46.3±2.1%, 0.87±0.01). Maximum vasoconstriction to Phe, AngII, and ET-1 was not significantly different between Norm-Preg (28.6±4.8%, 32.5±6.3%, 40±4.6%, respectively) and RUPP rats (27.8±5.9%, 34.4±4.3%, 38.8±4.1%, respectively). In contrast, the initial Phe, AngII, and ET-1 induced 340/380 ratio ([Ca\(^{2+}\)]\(_i\)) was reduced in RUPP (0.83±0.02, 0.82±0.02, 0.83±0.03, respectively) compared to Norm-Preg rats (0.95±0.04, 0.93±0.01, 0.92±0.02, respectively). Also, the [Ca\(^{2+}\)]\(_i\)-vasoconstriction relationship was similar in KCl-treated, but shifted to the left in Phe, AngII and ET-1 treated microvessels of RUPP compared to Norm-Preg rats. The lower agonist-induced [Ca\(^{2+}\)]\(_i\) signal of vasoconstriction and the leftward shift in the [Ca\(^{2+}\)]\(_i\)-vasoconstriction relationship in microvessels of RUPP compared to Norm-Preg rats suggest activation of [Ca\(^{2+}\)]\(_i\) sensitization pathway(s). The similarity in vasoconstriction in RUPP and Norm-Preg rats suggests that such [Ca\(^{2+}\)]\(_i\) sensitization pathway(s) may also provide a feedback effect on
Ca$^{2+}$ mobilization/homeostatic mechanisms to protect against excessive vasoconstriction in systemic microvessels during RUPP in late pregnancy.

**Key words:** resistance vessels, vascular smooth muscle, calcium, pregnancy, preeclampsia
**Introduction**

During normal pregnancy (Norm-Preg) increases in plasma volume, heart rate and renal blood flow as well as decreases in systemic vascular resistance, blood pressure (BP) and vascular reactivity to circulating vasoconstrictors are often observed (8,18,37,65). In contrast, in 5% to 7% of pregnancy, women develop a condition called preeclampsia, which is characterized by increased intravascular coagulation, proteinuria, increased systemic vascular resistance, and hypertension in pregnancy (HTN-Preg) (24,25,32,59,60,63,66). Although HTN-Preg is a major cause of maternal morbidity, fetal mortality and low-birth-weight (2,3,27), its exact mechanism has not been clearly identified.

Because of the difficulty to perform mechanistic studies in women with preeclampsia, animal models of HTN-Preg have been developed (1,13,14,44,57,58). Studies in these animal models have led to the concept that reduction in the uteroplacental perfusion pressure and the ensuing placental ischemia/hypoxia during late pregnancy may represent the initiating events that eventually lead to increased systemic vascular resistance and HTN-Preg. In support of this concept, studies have demonstrated that reduction of uterine perfusion pressure (RUPP) in late pregnant rats is associated with significant increases in renal vascular resistance and BP (5,6,14,26,63); however, the vascular and cellular mechanisms involved have not been clearly elucidated.

Vascular smooth muscle (VSM) contraction is triggered by an increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) due to initial Ca\(^{2+}\) release from the intracellular stores and maintained Ca\(^{2+}\) entry from the extracellular space (39-41,51). Ca\(^{2+}\) binds calmodulin to form a complex which activates myosin light chain kinase, causes myosin light chain phosphorylation, initiates actin-myosin interaction and produces VSM contraction (30,62). Previous studies in isolated VSM cells have suggested that phenylephrine (Phe)-induced contraction and [Ca\(^{2+}\)]\(_e\) are reduced in aortic VSM of female compared with male rats (51). Also, Phe-induced vascular contraction is reduced in aortic segments of Norm-Preg compared with virgin rats, but significantly enhanced in rat models of HTN-Preg produced...
by administration of the nitric oxide synthase (NOS) inhibitor L-NAME, or by inducing RUPP in late pregnancy (14,15,36,45). We have also shown that both contraction and [Ca\(^{2+}\)]\(_i\) are enhanced in renal arterial VSM cells isolated from RUPP rats compared with Norm-Preg rats (50). However, the vascular responses during pregnancy may not be uniform and may vary depending on the vascular bed studied and the vessel size down the arterial tree i.e. large, intermediate, small, and microvessels. The differences in the responses of various blood vessels can be related to differences in vasoconstrictor receptor distribution, receptor-coupling mechanisms, and postreceptor mechanisms particularly [Ca\(^{2+}\)]\(_i\) control mechanisms and [Ca\(^{2+}\)]\(_i\) sensitization pathways (7,31,45). Previous studies have shown that the mesenteric vascular resistance is elevated in rat models of HTN-Preg (6). Also, the mesenteric vascular resistance is reduced in Norm-Preg compared with nonpregnant normotensive rats (61,68,74), and in Norm-Preg compared with nonpregnant spontaneously hypertensive rats (12). However, little is known about the pregnancy-associated changes in the mechanisms of vasoconstriction in the small microvessels of the systemic circulation, which are directly relevant to the changes in BP. Also, in our previous studies we have examined the pregnancy-associated changes in vascular contraction and [Ca\(^{2+}\)]\(_i\) in response to only one agonist (15,49,50), making it difficult to appreciate whether the observed alterations are specific to a particular agonist/receptor, or represent changes in a common signaling mechanism downstream from receptor activation.

In the present study, we tested the hypothesis that the Ca\(^{2+}\)-dependent mechanisms of vasoconstriction in the systemic microvessels are differentially regulated under conditions of normal and RUPP in late pregnancy. We used small mesenteric microvessels isolated from the well-described RUPP rat model of HTN-Preg and control Norm-Preg rats to determine: 1) whether the mesenteric microvessel reactivity to four different vasoconstrictor stimuli is altered in RUPP compared with Norm-Preg rats; 2) whether the alterations in microvessel reactivity in RUPP compared with Norm-Preg rats reflect differences in the microvessel
[Ca$^{2+}$]; and 3) whether the alterations in microvessel reactivity in RUPP compared with Norm-Preg rats reflect differences in the microvessel vasoconstriction sensitivity to [Ca$^{2+}$].

**Material and Methods**

**Animals.** Time-pregnant (day 12) female Sprague-Dawley rats (12 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). The rats were housed in the animal facility and maintained on *ad libitum* standard rat chow and tap water in 12-hour light/dark cycle. All surgical procedures were performed using aseptic technique and proper anesthetics and analgesics in accordance with the NIH guide for the Care of Laboratory Animal Welfare Act, and the guidelines of the Animal Care and Use Committee at Harvard Medical School and the American Physiological Society.

**Protocol for RUPP.** On day 13 of pregnancy, pregnant rats destined to be in the RUPP group were anesthetized by inhalation of isoflurane, the abdominal cavity was opened by a midline incision, the lower abdominal aorta was exposed, and a silver clip (0.203 mm ID) was placed around the aorta above the iliac bifurcation as previously described (1,6,14,26,50). This procedure has been shown to reduce uterine perfusion pressure in the gravid rat by ~40% (20). Since compensation of blood flow to the placenta occurs through an adaptive increase in ovarian blood flow (53), a silver clip (0.1 mm ID) was also placed on the main uterine branches of both the right and left ovarian arteries. Norm-Preg rats were sham-operated. RUPP rats in which the clipping procedure resulted in maternal death or total resorption of the fetuses were excluded from the data analyses. Using the same RUPP protocol, the BP was ~25-35 mmHg greater in RUPP rats compared to Norm-Preg rats as previously reported (1,5,6,14,22,26,50,56).

**Tissue preparation.** On gestational day 19, the rats were euthanized by inhalation of CO$_2$. The abdominal cavity was opened, the pups and placentae were removed, the pups were
weighed and the litter size was recorded. The small intestine, adjacent mesentery and mesenteric arterial arcade were excised, and placed in ice-cold oxygenated Krebs solution. Small mesenteric arteries 3rd or 4th order were dissected free of surrounding adipose tissue under microscopic visualization.

**Pressurized microvessels.** A 1 to 2 mm microvessel segment was transferred to a temperature-controlled perfusion chamber (5 ml), mounted between two glass micropipettes (cannulae) and secured with 10-0 ophthalmic suture (Living Systems Instrumentation, Burlington, VT). The microvessel bath was placed on the stage of a Nikon inverted microscope with attached video camera. The lumen of the artery was filled with Krebs solution, one micropipette was clamped off, and the other micropipette was connected to a pressure servo control to maintain the intraluminal pressure at 60 mmHg. Applying the same constant pressure in the microvessels should limit potential fluctuations in endothelial cell production of vasodilators associated with changes in the microvessel pressure, flow and sheer stress. The Krebs solution within the microvessel was not renewed during pressurization; however, the microvessel was bathed in 5 ml Krebs and was continuously superfused with fresh Krebs at a rate of 1 ml/min using a peristaltic mini-pump (Master-Flex, Cole-Parmer, Vernon Hills, IL), which should maintain the ionic environment constant throughout the duration of the experiment. The temperature of Krebs solution was kept at 37°C. Drugs were added abluminally to the bath solution. Microvessels were unacceptable if they show leaks, or fail to produce >30% constriction to 96 mM KCl, or 80% dilation with sodium nitroprusside (10⁻⁵ M).

**Simultaneous measurement of microvessel diameter and [Ca²⁺]ᵢ.** The mesenteric microvessels were continuously monitored using a video camera connected to a TV monitor, and the microvessel diameter was measured using automatic edge-detection system (Crescent Electronics, Sandy, UT) and digitized at 1 Hz using a personal computer.
Snap-pictures of the microvessel were taken at specific time points using a digital camera (Cool-Snap, Photometrics, Tucson, AZ). For measurement of $[\text{Ca}^{2+}]_i$, microvessels were incubated in Krebs solution containing the $\text{Ca}^{2+}$ indicator fura-2/AM (5 µM) and pluronic F-127 (0.01%) for 60 min. The microvessel was excited alternately at 340 and 380 nm and the emitted light was collected at 510 nm using Felix Fluorescence data acquisition and analysis software (Photon Technology International, PTI, Birmingham, NJ). The 340/380 ratio was calculated and represented the changes in $[\text{Ca}^{2+}]_i$. The signal-to-noise ratio was improved by averaging 10 consecutive 340/380 fluorescence ratio readings.

The sensitivity of the contractile response to KCl and phenylephrine (Phe) has previously been published by our group and other laboratories in the aorta, uterine and mesenteric arteries of Norm-Preg and RUPP rats (5,6,14,23). Our initial experiments, demonstrated that the KCl and Phe-induced changes in $[\text{Ca}^{2+}]_i$ were rather small and we could not detect with confidence concentration-dependent changes in $[\text{Ca}^{2+}]_i$. Also, the angiotensin II (AngII) response in rat tissue is notoriously tachyphylactic, making it difficult to construct a cumulative concentration-constriction or concentration-$[\text{Ca}^{2+}]_i$ curve. Additionally, the endothelin-1 (ET-1) response was relatively slow in onset, particularly at low concentrations, and a cumulative-constriction response curve would require prolonged exposure to excitation light, which would cause significant photobleaching of fura-2 and affect the accuracy of $[\text{Ca}^{2+}]_i$ measurements. Therefore, in order to accurately compare the $[\text{Ca}^{2+}]_i$ dependent constriction induced by various agonists, we used maximal concentrations and a 10 min exposure time. The maximal concentrations of KCl, Phe, AngII and ET-1 used were based on previous reports from our laboratory and other groups, which have examined the concentration-constriction curves for KCl, Phe, AngII and ET-1 in the aorta, uterine, and mesenteric vessels of non-pregnant, Norm-Preg and RUPP rats (4-6,14,23). In all experiments, the microvessel was first stimulated with 96 mM KCl and the initial and maintained vasoconstriction and 340/380 ratio were measured. Once the constriction reached a plateau, the microvessel was washed with Krebs solution for 15 min.
The microvessel was then stimulated with either Phe (10^{-5} M), AngII (10^{-6} M), or ET-1 (10^{-7} M) and the initial and maintained vasoconstriction and 340/380 ratio were recorded, and used to construct the 340/380 ratio ([Ca^{2+}]_i)-vasoconstriction relationship for microvessels from Norm-Preg and RUPP rats.

**Solutions and drugs.** Normal Krebs solution contained (in mM): NaCl 120, KCl 5.9, NaHCO_3 25, NaH_2PO_4 1.2, dextrose 11.5, CaCl_2 2.5, MgCl_2 1.2, at pH 7.4, and bubbled with 95% O_2 and 5% CO_2. 96 mM KCl was prepared as Krebs solution with equimolar substitution of NaCl with KCl. Stock solutions of Phe, AngII and ET-1 (Sigma, St. Louis, MO) were prepared in distilled water. All other chemicals were of reagent grade or better.

**Statistical Analysis.** The data from Norm-Preg (n=10) and RUPP rats (n=9) were analyzed and presented as means±SEM. Student’s t-test for unpaired data was used for comparison of two means. Differences were considered statistically significant if P < 0.05.

**RESULTS**

**Effect of RUPP on Pups.** On the day of the experiment (day 19 of gestation), examination of the pup litter demonstrated that there was a significant reduction in litter size in RUPP (7.7±1.3 pups) compared with Norm-Preg (12.7±0.5 pups) (p=0.002). Also, the average pup weight was significantly reduced in RUPP rats (2.14±0.17 g) compared to that in Norm-Preg rats (2.68±0.12 g) (p=0.017).

**Resting Diameter and Basal [Ca^{2+}].** Cumulative data in unstimulated pressurized mesenteric microvessels demonstrated that the resting internal diameter was 240.8±11.2 μm in Norm-Preg rats and was not significantly different from that in RUPP rats (237.9±18.1 μm) (p=0.575). The thickness of the microvessel wall was 40.4±3.3μm in Norm-Preg rats and was not significantly different in RUPP rats (45.7±5.5μm). Also, the wall thickness to
luminal diameter was not significantly different in Norm-Preg compared with RUPP rats. On the other hand, the basal 340/380 ratio ([Ca\(^{2+}\)]\(_i\)) was significantly reduced in RUPP rats (0.73±0.03) compared to Norm-Preg rats (0.82±0.03) (P<0.05).

**Effect of KCl.** KCl (96 mM) caused a significant decrease in the diameter of microvessels of both Norm-Preg (Fig. 1A) and RUPP rats (Fig. 1B). The KCl-induced response demonstrated an initial maximum followed by maintained vasoconstriction (Fig. 1C, 1D). Also, in microvessels of both Norm-Preg and RUPP rats KCl caused a slight change in the 340 nm fura-2 fluorescence signal, a significant decrease in the 380 nm fluorescence signal (Fig. 1E, 1F) and an increase in the 340/380 fluorescence ratio (Fig. 1G, 1H), indicating simultaneous increase in [Ca\(^{2+}\)]\(_i\) during KCl-induced vasoconstriction. In both Norm-Preg and RUPP rats the KCl-induced [Ca\(^{2+}\)]\(_i\) preceded and reached peak before the maximum vasoconstriction (Table 1). Also, in microvessels of Norm-Preg and RUPP rats stimulated with KCl, the time to steady-state [Ca\(^{2+}\)]\(_i\) coincided with the time to steady-state vasoconstriction (Table 1). Cumulative data indicated that the KCl-induced initial and maintained vasoconstriction (Fig. 1I, 1J) and [Ca\(^{2+}\)]\(_i\) (Fig. 1K, 1L) were not significantly different between microvessels of Norm-Preg and RUPP rats.

**Effect of Phe.** Mesenteric microvessels of both Norm-Preg and RUPP rats showed an initial vasoconstriction followed by maintained decrease in diameter in response to the \(\alpha\)-adrenergic agonist Phe (10\(^{-5}\) M) (Fig. 2A, 2B). The Phe-induced vasoconstriction was preceded by an initial spike followed by smaller, but maintained increase in [Ca\(^{2+}\)]\(_i\) (Fig. 2C, 2D). In microvessels of both Norm-Preg and RUPP rats the Phe-induced [Ca\(^{2+}\)]\(_i\) preceded and reached peak before the maximum vasoconstriction (Table 1). Also, in microvessels of Norm-Preg and RUPP rats stimulated with Phe, the time to steady-state [Ca\(^{2+}\)]\(_i\) coincided with the time to steady-state vasoconstriction (Table 1). Cumulative data demonstrated no significant difference in Phe-induced initial or maintained vasoconstriction between Norm-
Preg and RUPP rats (Fig. 2E, 2F). In contrast, the Phe-induced initial and maintained [Ca^{2+}]_{i} presented as the 340/380 ratio was significantly reduced in RUPP compared with Norm-Preg rats (p< 0.05) (Fig. 2G, 2H).

**Effect of Ang II.** In mesenteric microvessels of both Norm-Preg and RUPP rats AngII (10^{-7} M) caused a rapid decrease in diameter that reached a maximum in ~30 seconds (Fig. 3A, 3B). AngII also caused an initial peak followed by maintained increase in [Ca^{2+}]_{i} in microvessels of both Norm-Preg and RUPP rats (Fig. 3C, 3D). In both Norm-Preg and RUPP rats the AngII-induced [Ca^{2+}]_{i} preceded and reached peak and steady-state levels before the maximum and steady-state vasoconstriction, respectively (Table 1). Cumulative data demonstrated no significant difference in the AngII-induced initial and maintained vasoconstriction (Fig. 3E, 3F). In contrast, the AngII-induced initial and maintained [Ca^{2+}]_{i} presented as the 340/380 ratio was significantly reduced (p< 0.05) in RUPP as compared to Norm-Preg rats (Fig. 3G, 3H).

**Effect of ET-1.** ET-1 (10^{-7} M) caused a relatively slow developing decrease in diameter of microvessels of Norm-Preg and RUPP rats that reached a steady state in ~45 sec. The ET-1 induced vasoconstriction was prolonged and could not be reversed despite washing the microvessels several times with Krebs solution (Fig. 4A, 4B). The ET-1-induced vasoconstriction was associated with an initial peak in [Ca^{2+}]_{i} followed by smaller, but maintained increase in [Ca^{2+}]_{i} (Fig. 4C, 4D). In both Norm-Preg and RUPP rats the ET-1-induced [Ca^{2+}]_{i} preceded and reached peak before the maximum vasoconstriction (Table 1). In contrast, in microvessels stimulated with ET-1 the time to steady-state [Ca^{2+}]_{i} lagged behind the time to steady-state vasoconstriction in both Norm-Preg (p=0.003) and RUPP rats (p=0.054) (Table 1). Cumulative data demonstrated no significant difference in ET-1 induced initial or maintained vasoconstriction between microvessels of Norm-Preg and
RUPP rats (Fig. 4E, 4F). In contrast, the ET-1-induced initial and maintained $[\text{Ca}^{2+}]_i$ was lower in RUPP as compared to Norm-Preg rats (Fig. 4G, 4H).

**[Ca$^{2+}$]$_i$-Vasoconstriction Relationship.** The initial and maintained 340/380 ratio ($[\text{Ca}^{2+}]_i$) and vasoconstriction in response to KCl, Phe, Ang II, and ET-1 were used to construct the $[\text{Ca}^{2+}]_i$-vasoconstriction relationship in microvessels of Norm-Preg and RUPP rats. Both the initial and maintained KCl $[\text{Ca}^{2+}]_i$-vasoconstriction relationship were not different between Norm-Preg and RUPP rats. In contrast, the initial and maintained $[\text{Ca}^{2+}]_i$-vasoconstriction relationship for Phe, AngII or ET-1 was shifted to the left in RUPP rats as compared to Norm-Preg rats (Fig. 5).

**DISCUSSION**

The main findings of the present study are: 1) the maximal mesenteric microvessel vasoconstriction to KCl and the more physiological vasoconstrictor stimuli Phe, AngII and ET-1 is not different between RUPP and Norm-Preg rats; 2) the KCl-induced $[\text{Ca}^{2+}]_i$ is not different, but the basal and agonist-induced initial and maintained $[\text{Ca}^{2+}]_i$ are reduced in RUPP compared with Norm-Preg rats, and 3) the $[\text{Ca}^{2+}]_i$-vasoconstriction relationship for KCl is similar, while that for Phe, AngII or ET-1 is shifted to the left (enhanced) in microvessels of RUPP compared with Norm-Preg rats.

Previous studies have shown that the mesenteric vascular resistance is elevated in rat models of HTN-Preg (6). Studies have also shown that the mesenteric vascular resistance is reduced in Norm-Preg compared with nonpregnant normotensive rats (61,68,74), and in Norm-Preg compared with nonpregnant spontaneously hypertensive rats (12). Studies have also shown that the vascular reactivity to electrical stimulation or intra-arterial noradrenaline, AngII and arginine vasopressin are decreased in the *in situ* blood perfused mesenteries of Norm-Preg rats compared with nonpregnant controls (11). Other studies in the *in situ* blood
perfused mesenteric resistance vessels of 18-20 day pregnant spontaneously hypertensive rats have shown much lower vascular response to electrical stimulation or intra-arterial noradrenaline than either pregnant or nonpregnant spontaneously hypertensive rats (12). Although the in situ blood-perfused mesenteries could provide important physiological vascular reactivity information, they may not allow further investigation of underlying cellular mechanisms, particularly measurement of \([\text{Ca}^{2+}]_i\).

One goal of the present study was to investigate the mechanisms of vasoconstriction in small systemic microvessels of Norm-Preg and RUPP rats. Arteries of internal diameter <300 µm are by and large considered resistance vessels (47,48,75). Specifically, the small mesenteric feed arteries and microcirculatory vessels have been used in several studies as representative of resistance vessels (6,21,52). These small resistance size arteries have significant myogenic tone that primarily maintains constant blood flow and provide a baseline diameter that is modulated by vasoconstrictors and vasodilators (10,16,75). Therefore, we used isolated mesenteric microvessels to determine the \([\text{Ca}^{2+}]_i\)-signaling mechanism underlying the vascular changes in Norm-Preg and RUPP rats. The average internal diameter of the mesenteric microvessels used in the present study was 241 in Norm-Preg and 238 µm in RUPP rats, well in the range of resistance arteries. Also, all microvessels tested produced ~45% constriction in response to KCl, and significant vasoconstriction to three different physiological agonists namely Phe, AngII and ET-1, confirming viability of the microvessel preparation. Furthermore, during stimulation by KCl and other agonists the time to peak \([\text{Ca}^{2+}]_i\) always preceded the time to maximum vasoconstriction (Table 1), supporting the contention that the increased \([\text{Ca}^{2+}]_i\) triggers the vasoconstriction.

Previous studies have shown that RUPP in late pregnant rats is associated with significant increases in renal vascular resistance and BP (1,14,22,26). Also, we have shown that Phe-induced vascular contraction is greater in aortic strips isolated from RUPP compared with Norm-Preg rats (14). Although the differences in aortic contraction were
partially-related to reduced endothelium-dependent nitric oxide-mediated vascular relaxation in RUPP rats, differences in contraction were still observed in endothelium-denuded aortic strips of RUPP compared with Norm-Preg rats, suggesting additional differences in the mechanisms of aortic VSM contraction (14). In support of this view, studies have shown enhanced Phe-induced contraction in isolated uterine arteries from RUPP or transgenic preeclampsia rats compared with Norm-Preg rats (5,70).

In search for the cellular mechanisms involved in the enhanced vasoconstriction during HTN-Preg, our previous experiments on the aorta have shown that the Phe- and caffeine-induced contraction in Ca^{2+}-free solution are not different in RUPP rats as compared to Norm-Preg rats, suggesting that the IP_{3}-sensitive and the Ca^{2+}-induced Ca^{2+} release mechanisms from the intracellular stores are not different between the RUPP rat model of HTN-Preg and Norm-Preg rats (23). On the other hand, the aortic contractile response to membrane depolarization by KCl, which stimulates Ca^{2+} entry from the extracellular space, is reduced in Norm-Preg compared with virgin rats, but significantly enhanced in RUPP rats (15,23,36). Also, our studies in isolated renal arterial VSM cells have shown that the basal and AngII-stimulated [Ca^{2+}]_i are reduced in Norm-Preg compared with virgin rats, but significantly elevated in RUPP rats (50). These data have suggested that the Ca^{2+} entry mechanisms of vascular contraction are enhanced in the aorta and renal artery of RUPP rats as compared to Norm-Preg rats.

Based on previous measurements of VSM contraction and Ca^{2+} in the aorta, renal and uterine artery (5,15,36,50), we hypothesized that the Ca^{2+}-dependent mechanisms of vasoconstriction are most likely enhanced in small mesenteric microvessels of RUPP compared with Norm-Preg rats. Contrary to our prediction, the KCl-induced vasoconstriction and [Ca^{2+}]_i were not different in mesenteric microvessels of RUPP and Norm-Preg rats. Because KCl largely stimulates Ca^{2+} influx through voltage-gated Ca^{2+} channels, the present data suggest that this Ca^{2+} entry mechanism of vasoconstriction is not different in mesenteric microvessels of Norm-Preg and RUPP rats. We should note that while the KCl-
induced response is thought to be mainly due to Ca$^{2+}$ entry from the extracellular space, the KCl-induced Ca$^{2+}$ entry can also activate Ca$^{2+}$ release from internal stores by Ca$^{2+}$-induced Ca$^{2+}$ release mechanism, and the contribution of this mechanism to the observed KCl response can not be ruled out.

The present data also demonstrated that the mesenteric microvessel reactivity to Phe was not significantly different between RUPP and Norm-Preg rats. Because different parts of the circulation may have different distribution of $\alpha$-adrenergic receptors, we hypothesized that the lack of change in the responsiveness to Phe in mesenteric microvessels of RUPP rats as compared to the previously reported enhanced sensitivity to Phe in the aorta and uterine artery may be related to decreased amount of $\alpha$-adrenergic receptors in the mesenteric vessels. If this is the case, then the mesenteric microvessels of RUPP rats should be more responsive to other agonists/receptors. Thus, a second goal of the present study was to investigate whether the differences in the mechanisms of vasoconstriction in systemic microvessels of Norm-Preg and RUPP rats are specific to a particular agonist/receptor or represent difference in a common signaling pathway downstream from receptor activation.

AngII, which stimulates angiotensin type 1 receptor in VSM, caused significant vasoconstriction of mesenteric microvessels that was similar in RUPP and Norm-Preg rats. Also, ET-1, which stimulates $\text{ET}_A$ and perhaps $\text{ET}_{B2}$ receptor in VSM, induced similar vasoconstriction in RUPP and Norm-Preg rats. Nevertheless, similar to the Phe response, the AngII- and ET-1-induced [Ca$^{2+}$], was lower in RUPP compared with Norm-Preg rats. These data suggest that the decreased [Ca$^{2+}$], signaling of vasoconstriction in the RUPP rats in response to the $\alpha$-adrenergic agonist Phe, is shared by other agonists such as AngII and ET-1 which act on different sets of receptors. Alternatively, the decreased [Ca$^{2+}$], signaling of vasoconstriction in response to Phe, AngII and ET-1 in RUPP compared with Norm-Preg rats may represent a difference in a common [Ca$^{2+}$], regulatory pathway downstream from receptor activation. Agonists such as Phe, AngII and ET-1 are known to
activate Ca\textsuperscript{2+} influx through ligand-gated and store-operated Ca\textsuperscript{2+} channels in VSM (17). The decreased [Ca\textsuperscript{2+}]\textsubscript{i} signaling of vasoconstriction in response to Phe, AngII and ET-1 in RUPP compared with Norm-Preg rats may therefore reflect reduced expression/activity of ligand-gated and/or store-operated Ca\textsuperscript{2+} channels. However, if [Ca\textsuperscript{2+}]\textsubscript{i} is the sole regulator of the microvessel vasoconstriction, then the reduced [Ca\textsuperscript{2+}]\textsubscript{i} in microvessels of RUPP rats should be associated with reduced vasoconstriction. This is not the case, as the microvessel vasoconstriction was similar in magnitude in RUPP rats and Norm-Preg rats, suggesting activation of other control or signaling mechanisms in addition to [Ca\textsuperscript{2+}]\textsubscript{i}.

An important observation is that the basal [Ca\textsuperscript{2+}]\textsubscript{i} was lower in mesenteric microvessels of RUPP rats compared with Norm-Preg rats. Ca\textsuperscript{2+} homeostasis is controlled by Ca\textsuperscript{2+} extrusion mechanisms in the plasma membrane including the Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (40). The decreased basal [Ca\textsuperscript{2+}]\textsubscript{i} in microvessels of RUPP rats may be related to enhanced Ca\textsuperscript{2+} extrusion mechanisms. Similarly, the reduced [Ca\textsuperscript{2+}]\textsubscript{i} response to vasoconstrictor agonists in RUPP rats could be partly explained by increased activity of Ca\textsuperscript{2+} extrusion mechanisms. Another possibility is that the vasoconstriction sensitivity to [Ca\textsuperscript{2+}]\textsubscript{i} is altered in microvessels of RUPP rats. We found that the [Ca\textsuperscript{2+}]\textsubscript{i}-vasoconstriction relationship was similar in KCl-stimulated microvessels of Norm-Preg and RUPP rats. In contrast, the [Ca\textsuperscript{2+}]\textsubscript{i}-vasoconstriction relationship was enhanced in Phe-, AngII- and ET-1 stimulated microvessels of RUPP compared with Norm-Preg rats. These findings suggest activation of a [Ca\textsuperscript{2+}]\textsubscript{i} regulatory pathway that increases the myofilament sensitivity to [Ca\textsuperscript{2+}]\textsubscript{i}. Several studies have shown that in addition to the role of Ca\textsuperscript{2+}, calmodulin and myosin light chain kinase, Rho-kinase and mitogen-activated protein kinase may contribute to VSM contraction (28-30,63,73). Also, protein kinase C (PKC) has been suggested to play an important role in the regulation of VSM contraction, in part by increasing the [Ca\textsuperscript{2+}]\textsubscript{i} sensitivity of the contractile proteins (38,41,54,35,62,72). Additionally, previous studies in rat small mesenteric arteries have shown that noradrenaline-induced Ca\textsuperscript{2+} sensitization is associated with increased myosin light chain phosphorylation and suggested decreased
myosin light chain phosphatase activity that is mediated through PKC (9,55). Collectively, the present and previous studies suggest that while Ca\textsuperscript{2+}-dependent myosin light chain phosphorylation is a major regulator of vasoconstriction in both Norm-Preg and RUPP rats, other [Ca\textsuperscript{2+}] sensitization pathways such as PKC or Rho-kinase may be involved in the regulation of vasoconstriction in microvessels of RUPP rats.

If activation of a Ca\textsuperscript{2+} regulatory pathway such as PKC causes an increase in the vasoconstriction sensitivity to [Ca\textsuperscript{2+}], then the question is why the initial and maintained vasoconstriction are similar in microvessels of Norm-Preg and RUPP rats? Studies have suggested that PKC may activate feedback mechanisms involving uncoupling of the surface receptors from the GTP-binding protein, inhibition of phospholipase C, inhibition of Ca\textsuperscript{2+} mobilization via the Ca\textsuperscript{2+} release or Ca\textsuperscript{2+} entry channels, activation of Ca\textsuperscript{2+} extrusion mechanisms, and phosphorylation and inhibition of myosin light chain kinase (35,62). Interestingly, during microvessel stimulation with most of the agonists tested in the present study the time to steady-state [Ca\textsuperscript{2+}], preceded or coincided with the time to steady-state vasoconstriction, suggesting that [Ca\textsuperscript{2+}] remains a major determinant of vasoconstriction during steady-state. A clear exception was the ET-1 response in which the time to steady-state [Ca\textsuperscript{2+}], lagged behind the time to steady-state vasoconstriction. We have previously shown that ET-1 promotes VSM contraction via activation of PKC (46), raising the possibility that an ET-1 induced activation of PKC during maximal contraction would cause feedback control of Ca\textsuperscript{2+} entry, and therefore a delay in the [Ca\textsuperscript{2+}], steady-state. PKC is a family of Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent isoforms that exhibit different enzyme properties, substrates, functions and sub-cellular distributions in various blood vessels (35,55,62), and therefore, activation of PKC may have different effects in various vascular beds particularly during pregnancy. We have previously shown that the vascular sensitivity to Ca\textsuperscript{2+} entry was enhanced in the aorta of L-NAME treated compared with control Norm-Preg rats (15). We have also shown that the activity of the Ca\textsuperscript{2+}-dependent \(\alpha\)-PKC and the Ca\textsuperscript{2+}-independent \(\delta\)-PKC is enhanced in L-NAME treated
compared with control Norm-Preg rats (33,34). Although our previous data point to PKC as a possible regulator of Ca\textsuperscript{2+} signaling in the L-NAME treated rat model of HTN, the specific role of PKC or other Ca\textsuperscript{2+} sensitization pathways such as Rho kinase in the RUPP model of HTN-Preg is unclear at the present time and should be further examined in future studies.

We should note that previous wire myography studies in 1\textsuperscript{st} order mesenteric arteries have shown enhanced vasoconstrictor responses to a range of KCl, Phe and AngII concentrations in RUPP rats compared with Norm-Preg rats (6). The differences in the results could be related to differences in recording techniques (wire myography vs. pressurized microvessels) or in the size of the vessels studied (1\textsuperscript{st} order arteries as compared to 3\textsuperscript{rd} to 4\textsuperscript{th} branch mesenteric microvessels). Our results in the RUPP rats are consistent with studies in human vessels, which demonstrated no significant differences in basal myogenic tone or constrictor responses to KCl, Phe or AngII in subcutaneous resistance arteries from women with preeclampsia compared with those from Norm-Preg women (69,71). It has been suggested that the vasoconstrictor sensitivity of arteries may be different in isobaric versus isometric conditions for reasons related to both the mounting technique and mechanical loading (9,67). It has also been suggested that conditions associated with medial hypertrophy might result in a higher maximal tension development to vasoconstrictors in isometric arteries, while isobaric arteries may show similar maximal constrictions as long as the pressure load is not too high (69).

Thus the Ca\textsuperscript{2+} regulatory mechanisms in mesenteric microvessels appear to be different from those previously demonstrated in the aorta, uterine arteries or main mesenteric arteries of RUPP rats as compared to Norm-Preg rats (5,6,14,23). Whether the difference represents different adaptation mechanisms in the mesenteric microvessels as compared to other vascular beds or large conduit vessels needs to be further examined. The microvessel vasoconstriction to three different physiological agonists acting through different receptors is associated with smaller increases in [Ca\textsuperscript{2+}]\textsubscript{i} in RUPP as compared to Norm-Preg rats. The smaller [Ca\textsuperscript{2+}]\textsubscript{i} signaling of vasoconstriction in microvessels of RUPP
compared to Norm-Preg rats suggests activation of additional Ca$^{2+}$ regulatory pathway(s) that increase the vasoconstriction sensitivity to [Ca$^{2+}$]. The similarity in the initial and maintained vasoconstriction in RUPP and Norm-Preg rats suggests that such Ca$^{2+}$ regulatory pathway(s) may have feedback effect on Ca$^{2+}$ mobilization/homeostatic mechanisms to protect against excessive vasoconstriction in systemic microvessels during RUPP in late pregnancy.

**Perspective**

Preeclampsia is associated with increased total vascular resistance, which is thought to cause generalized organ hypoperfusion and multisystem disorder. Vascular resistance is determined by vascular tone, which depends on vascular smooth muscle [Ca$^{2+}$]$_i$ and the Ca$^{2+}$-sensitivity of the contractile proteins. Studying the vascular reactivity and [Ca$^{2+}$]$_i$ in animal models of HTN-Preg should help to elucidate the mechanisms of preeclampsia in women. Studies in the aorta, uterine and main mesenteric arteries suggest an increase in vascular reactivity in rat models of HTN-Preg (5,6,14,23). In contrast, the present study suggests no difference in reactivity of mesenteric microvessels of RUPP rats compared with Norm-Preg rats, a finding that is consistent with previously reported lack of difference in vascular reactivity in subcutaneous resistance arteries from preeclamptic and Norm-Preg women (69,71). The present results suggest that the vascular responses during HTN-Preg are not uniform and highlight the importance of studying other vascular beds including the renal, coronary and cerebral resistance arteries in future studies. The study also highlight the importance of measuring [Ca$^{2+}$]$_i$ and Ca$^{2+}$-sensitization pathways in blood vessels to demonstrate potential abnormalities in the underlying cellular mechanisms, despite apparent lack of changes in vascular reactivity. Many factors could affect vascular [Ca$^{2+}$]$_i$ in preeclampsia, including depolarization of the membrane potential, maladjustment of Ca$^{2+}$ influx across the plasma membrane and Ca$^{2+}$ release from intracellular stores in addition to abnormal transport of sodium and magnesium, and alteration of Ca$^{2+}$ metabolism and
plasma Ca$^{2+}$ (19,42,71), and these factors should be thoroughly examined in future investigations.

**ACKNOWLEDGEMENTS**

This work was supported by grants from National Heart, Lung, and Blood Institute (HL-65998 and HL-70659).

**REFERENCES**


Table 1. Time-to-Peak and Time-to-Steady-State Vasoconstriction and [Ca\(^{2+}\)] in Response to KCl (96 mM), Phe (10\(^{-5}\) M), AngII (10\(^{-7}\) M) and ET-1 (10\(^{-7}\) M) in Norm-Preg and RUPP rats.

<table>
<thead>
<tr>
<th></th>
<th>Norm-Preg</th>
<th></th>
<th></th>
<th></th>
<th>RUPP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KCl</td>
<td>Phe</td>
<td>AngII</td>
<td>ET-1</td>
<td>KCl</td>
<td>Phe</td>
<td>AngII</td>
</tr>
<tr>
<td>Time-to-Maximum</td>
<td>38.4±2.4</td>
<td>34.4±6.2</td>
<td>33.3±5.8</td>
<td>46.5±6.5</td>
<td>26.8±4.3</td>
<td>29.4±9.2</td>
<td>33.8±7.8</td>
</tr>
<tr>
<td>Constriction (sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-to-Peak</td>
<td>19.8±3.1</td>
<td>10.8±0.9</td>
<td>9.3±1.9</td>
<td>13.5±1.5</td>
<td>22.1±6.6</td>
<td>12.6±2.4</td>
<td>12.8±1.5</td>
</tr>
<tr>
<td>[Ca(^{2+})] (sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-to-Steady-State</td>
<td>106.4±20.1</td>
<td>86.6±15.7</td>
<td>125±20.7</td>
<td>97±9</td>
<td>115.8±12.1</td>
<td>88.8±19.9</td>
<td>135±12.1</td>
</tr>
<tr>
<td>Constriction (sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-to-Steady-state</td>
<td>105.3±8.8</td>
<td>85.8±14.2</td>
<td>108.7±6.9</td>
<td>148.8±11.8</td>
<td>114.3±12.9</td>
<td>89.5±16.1</td>
<td>92.2±2.2</td>
</tr>
<tr>
<td>[Ca(^{2+})] (sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

**Fig. 1.** Effect of KCl (96 mM) on vasoconstriction and [Ca²⁺]ᵢ in mesenteric microvessels of Norm-Preg and RUPP rats. Images of fura-2 loaded microvessel from Norm-Preg (A) and RUPP rat (B) were taken at rest and after stimulation with KCl. Simultaneous measurements of diameter, 340 and 380 nm fluorescence signal, and 340/380 ratio were recorded in the isolated microvessel of Norm-Preg (C,E,G) and RUPP rat (D,F,H). Bar graphs represent means±SEM of vasoconstriction and [Ca²⁺]ᵢ measurements in 10 microvessels of Norm-Preg (I,K) and 9 microvessels of RUPP rats (J,L).

**Fig. 2.** Effect of Phe (10⁻⁵ M) on vasoconstriction and [Ca²⁺]ᵢ in mesenteric microvessels of Norm-Preg and RUPP rats. Simultaneous measurements of Phe-induced changes in diameter and 340/380 fluorescence ratio were recorded in isolated microvessels from Norm-Preg (A,C) and RUPP rat (B,D). Bar graphs represent means±SEM of vasoconstriction and [Ca²⁺]ᵢ measurements in 10 microvessels of Norm-Preg (E,G) and 9 microvessels of RUPP rats (F,H).

* † [Ca²⁺]ᵢ (340/380 ratio) measurements in RUPP rats are significantly different (p<0.05) from corresponding measurements in Norm-Preg rats.

**Fig. 3.** Effect of AngII (10⁻⁷ M) on vasoconstriction and [Ca²⁺]ᵢ in mesenteric microvessels of Norm-Preg and RUPP rats. Simultaneous measurements of AngII-induced changes in diameter and 340/380 fluorescence ratio were recorded in isolated microvessels from Norm-Preg (A,C) and RUPP rat (B,D). Bar graphs represent means±SEM of vasoconstriction and [Ca²⁺]ᵢ measurements in 10 microvessels of Norm-Preg (E,G) and 9 microvessels of RUPP rats (F,H).

* † [Ca²⁺]ᵢ (340/380 ratio) measurements in RUPP rat are significantly different (p<0.05) from corresponding measurements in Norm-Preg rats.
Fig. 4. Effect of ET-1 (10^{-7} M) on vasoconstriction and [Ca^{2+}]_{i} in mesenteric microvessels of Norm-Preg and RUPP rats. Simultaneous measurements of ET-1-induced changes in diameter and 340/380 fluorescence ratio were recorded in isolated microvessels from Norm-Preg (A,C) and RUPP rat (B,D). Bar graphs represent means±SEM of vasoconstriction and [Ca^{2+}]_{i} measurements in 10 microvessels of Norm-Preg (E,G) and 9 microvessels of RUPP rats (F,H).

* † [Ca^{2+}]_{i} (340/380 ratio) measurements in RUPP rats are significantly different (p<0.05) from corresponding measurements in Norm-Preg rats.

Fig. 5. [Ca^{2+}]_{i}-vasoconstriction relationship in mesenteric microvessels from Norm-Preg and RUPP rats. Mesenteric microvessels from Norm-Preg and RUPP rats were stimulated with either KCl (96 mM), Phe (10^{-5}M), AngII (10^{-7}M) or ET-1 (10^{-7}M) and the initial (A) and maintained (B) changes in vasoconstriction and [Ca^{2+}]_{i} (340/380 ratio) were used to construct a [Ca^{2+}]_{i}-vasoconstriction relationship. The initial and maintained Phe, AngII and ET-1 induced [Ca^{2+}]_{i}-vasoconstriction relationships were shifted to the left (less [Ca^{2+}]_{i}) in RUPP rats compared to Norm-Preg rats.

* † [Ca^{2+}]_{i} (340/380 ratio) measurements in RUPP rats are significantly different (p<0.05) from corresponding measurements in Norm-Preg rats.
Effect of Phe (10^{-5} M)

**Pregnant**

- **A**
  - Diameter (μm)
  - Time: 2 min

**RUPP**

- **B**
  - Diameter (μm)

**C**

- Ratio (340/380)

**D**

- Ratio (340/380)

**E**

- Constriction (%)
  - Initial: 30%
  - Maintained: 25%

**F**

- Constriction (%)
  - Initial: 30%
  - Maintained: 20%

**G**

- Ratio (340/380)
  - Initial: 1.0
  - Maintained: 0.9

**H**

- Ratio (340/380)
  - Initial: 0.9
  - Maintained: 0.8

- * Phe
- † Phe

Additional notes:

- AB
- C
- D
- E
- G
- H
**Effect of AngII (10^{-6} M)**

**A**

Pregnant

Diameter (µm)

0.6

0.8

1.0

2 min

**B**

RUPP

Diameter (µm)

0.6

0.8

1.0

**C**

Ratio (340/380)

0.6

0.8

1

**D**

Ratio (340/380)

0.6

0.8

1

**E**

Constriction (%)

20

30

40

50

Initial

Maintained

**F**

Constriction (%)

20

30

40

50

Initial

Maintained

**G**

Ratio (340/380)

0.6

0.8

1

Initial

Maintained

**H**

Ratio (340/380)

0.6

0.8

1

Initial

Maintained

* †
Effect of ET-1 (10^{-7} M)

Pregnant

ET-1  W

Diameter (µm)

0.6  0.8  1.0

Effect of ET-1 (10^{-7} M)

ET-1  W

Ratio (340/380)

Initial Maintained Constriction (%)