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# Vascular Responses to Angiotensin-(1-7) During the Estrous Cycle

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**Mesenteric arteries (230–290 µm) were isolated from virgin female rats at diestrous and proestrous phases of the estrous cycle and from ovariectomized (OVX) rats with or without estrogen ($E_2$) replacement (17β-estradiol, 7.5 + 5 mg pellets, 21 d release). Arteries were mounted in a pressurized myograph system. Angiotensin-(1-7) [Ang-(1-7)] concentration-dependent responses ($10^{-10}$–$10^{-5}$ *M*) were determined in arteries preconstricted with endothelin-1 ($10^{-7}$ *M*). Mesenteric arteries were pretreated with the specific Ang-(1-7) antagonist, D-[Ala$^7$]-Ang-(1-7) ($10^{-7}$ *M*) to assess the Ang-(1-7) receptor-mediated dilator effect. Ang-(1-7) did not dilate mesenteric arteries from virgin rats at diestrus and placebo-treated OVX female rats as compared to the time control; however, Ang-(1-7) elicited a modest dilation at proestrus as compared to diestrus, which reached statistical significance at $10^{-8}$ *M* concentrations. Ang-(1-7) caused a concentration-dependent vasodilation in mesenteric arteries of females with $E_2$ replacement, with an $EC_{50}$ of 21 n*M*. D-[Ala$^7$]-Ang-(1-7) blocked the vasodilator effect of Ang-(1-7). Our results demonstrate that during proestrus Ang-(1-7) elicits modest vasodilation as compared to diestrus, but lacks vasodilatory properties in vessels from diestrous and ovariectomized rats. Estrogen replacement restores a significant dilator response to Ang-(1-7) in OVX rats that is mediated by a D-[Ala$^7$]-Ang-(1-7) sensitive site.**

**Key Words:** Hypertension; renin–angiotensin system; vasodilation; estrogen replacement.

## Introduction

Angiotensin-(1-7) [Ang-(1-7)] is a vasodilator peptide that opposes the vasoconstrictor actions of angiotensin II (Ang II) *(1,2)*. In [(m*Ren2*)27] transgenic hypertensive rats, we found that estrogen treatment enhanced the in vivo Ang-(1-7) depressor response in ovariectomized (OVX) transgenic animals, and this occurred in conjunction with reduced levels of plasma Ang II and increased levels of Ang-(1-7) *(3)*. Thus, we suggested that estrogen may be protective by amplifying the vasodilator actions of Ang-(1-7), while reducing the formation of Ang II. This possibility was further explored in pregnancy, a condition where endogenous levels of estrogen and Ang-(1-7) are markedly increased *(4)*. In those studies we reported that Ang-(1-7) had a marked vasodilator action in the mesenteric arteries of pregnant rats. However, we also observed that Ang-(1-7) was without effect in vessels taken from females in the diestrous phase of the estrous cycle *(4)*. This raised the question of whether the dilator response to Ang-(1-7) in rats is modulated by changes in ovarian endocrine activity during the estrous cycle.

In this study we compared the Ang-(1-7) vasodilator response in vessels from virgin rats at diestrus and proestrus to determine if Ang-(1-7)'s dilator response varied with the estrous cycle. In addition, we compared these findings with those observed in OVX rats with and without estrogen ($E_2$) replacement.

## Results

Cytological analysis of the vaginal smears of virgin female rats was employed to assess the phases of the estrous cycle. The results were confirmed by concurrent measures of plasma 17β-estradiol values. Plasma 17β-estradiol concentration in virgin females at proestrus (PE) [72.9 ± 21.9 pmol/L ($n$ = 11)] [$n$ = 2 at the minimun detectable level of the assay (MDL) of <14.8 pmol/L and $n$ = 9 at 85.1 ± 25.2 pmol/L] was significantly elevated when compared to virgin female rats at diestrus (DE) [20.8 ± 2.5 pmol/L ($n$ = 5), $p < 0.05$ ($n$ = 4 at the MDL and $n$ = 1 at 28.3 pmol/L). In placebo-treated OVX (OVX-PL) female animals plasma 17β-estradiol levels averaged 24.7 ± 3.6 pmol/L ($n$ = 14); a value not different from those measured at DE ($n$ = 10 at the MDL and $n$ = 4 at 40.6 ± 9 pmol/L). On the other hand, plasma estradiol levels were 2298 ± 168 pmol/L in $E_2$ replaced OVX rats (OVX-$E_2$) ($n$ = 14), a value significantly higher than those found in either OVX-PL female or virgin rats at DE and PE, $p < 0.001$. There was no difference in the body

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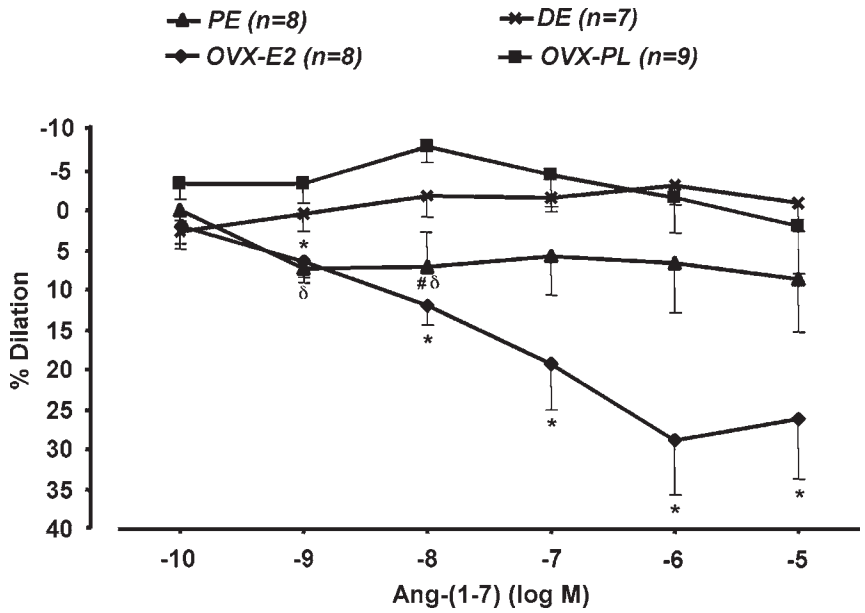


**Fig. 1.** The spontaneous relaxation of the time control was subtracted and the concentration–response curve to Ang-(1-7) ($10^{-10}$–$10^{-5}$ *M*) in mesenteric arteries from virgin female rats at diestrus (DE) and proestrus (PE) and OVX rats with and without 17β-estradiol (OVX-$E_2$ and OVX-PL replacement). The responses are expressed as the percent dilation relative to the constricted vessel diameter induced by endothelin-1. Differences among the means were evaluated using one-way analysis of variance (ANOVA) followed by Newman-Keuls or Dunn's multiple comparisons test. $^{*}p < 0.05$ for OVX-$E_2$ vs OVX-PL; $^{\#}p < 0.05$ for PE vs DE; $^{\delta}p < 0.05$ for PE vs OVX-PL.

weight of virgin rats at PE (251 ± 5.01 g) and DE (245 ± 5.9 g) (ns), but ovariectomy significantly increased body weight, 320 ± 9.8 g when compared either to OVX-E2 rats (236 ± 3.8 g) or virgin rats at both PE and DE ($p < 0.001$).

### *Effect of Ang-(1-7) on Vascular Reactivity*

The contribution of estrogen to the vascular reactivity to Ang-(1-7) during the estrous cycle was addressed by determining the Ang-(1-7)-mediated concentration-dependent dilator response in isolated mesenteric arteries of virgin female rats at diestrous and proestrous phases of the estrous cycle. In another group of animals the effects of $E_2$ replacement was determined in OVX female rats. There was no significant difference in baseline vessel diameter among the groups. Superfusion of $10^{-7}$ *M* ET-1 significantly reduced the luminal diameter by 71.1 ± 0.7% (from 252 ± 2 to 73 ± 2 μm, $n = 81$, $p < 0.001$), similar to previously reported studies *(4,5)*.

Figure 1 illustrates the dilator response of each group after the influence of the spontaneous dilation of the time control was removed and allows for comparisons among the groups. Ang-(1-7) had no effect on the diameter of ET-1 preconstricted mesenteric arteries in virgin rats at DE, confirming our previously reported results *(4,5)*, and in OVX rats. On the other hand, Ang-(1-7) elicited a modest dilation in mesenteric vessels from virgin rats at PE, reaching statistical significance at $10^{-8}$ *M* as compared to diestrus and at $10^{-9}$ *M* and $10^{-8}$ *M* as compared to OVX. With 17β-estradiol replacement, Ang-(1-7) caused a significant concentration-dependent ($10^{-10}$ to $10^{-5}$ *M*) dilation of mesenteric vessels ($p < 0.01$), reaching a maximum vasodilator plateau at $10^{-6}$ *M* ($EC_{50}$ of 21 n*M*). There was a significantly greater dilator response between OVX + $E_2$ as compared to OVX with placebo treatment.

Pretreatment with the specific Ang-(1-7) receptor antagonist, D-Ala$^7$-Ang-(1-7), blocked the dilator response of OVX+E2 treated rats (Fig. 2). It had no effect in rats at diestrus, proestrus, and in OVX+placebo treated rats (not shown).

## Discussion

During the normal estrous cycle there was no dilatory response to Ang-(1-7) during diestrus and in surgically ovariectomized rats. Rats at proestrus showed a slight increased vasodilator effect when compared to rats at diestrus and ovariectomized rats. Estrogen replacement restores the vasodilator effect of Ang-(1-7) of ovariectomized rats. This response was blocked by the specific Ang-(1-7) receptor antagonist *(6)*, D-Ala$^7$-Ang-(1-7). Our results suggest that Ang-(1-7) vasodilator response in female rats is modulated by circulating estrogen levels.

These findings confirm our previous report showing a lack of Ang-(1-7) vasodilator response in virgin rats at diestrus but a marked vasodilation of mesentery vessels during pregnancy *(4)*. The vasodilation found during pregnancy agrees with the vasodilation observed in this study with estrogen replacement, although the levels of estradiol achieved with estrogen replacement were substantially higher than

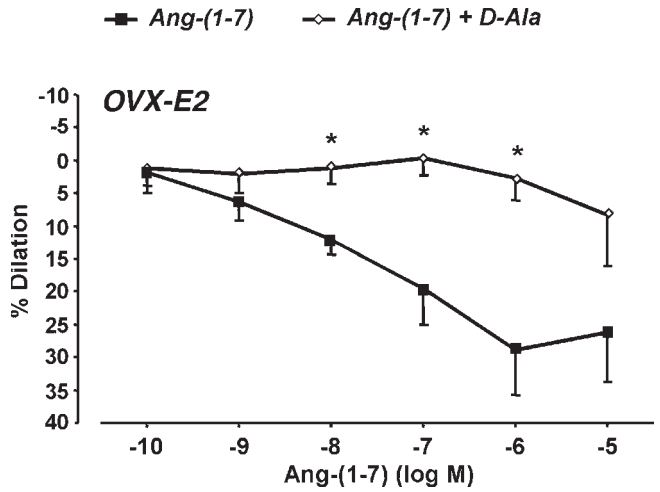


**Fig 2.** Lumen diameter of mesenteric arteries from OVX female rats with 17β-estradiol (OVX-$E_2$) replacement was assessed after addition of Ang-(1-7) ($10^{-10}$–$10^{-5}$ *M*) in the presence or absence of $10^{-7}$ *M* D-[$Ala^7$]-Ang-(1-7) pretreatment. Differences among the means were evaluated using one-way analysis of variance (ANOVA) followed by Newman–Keuls or Dunn's multiple comparisons test. *$p < 0.05$ compared to Ang-(1-7) alone.

those found with pregnancy and reflect pharmacological treatment.

Proestrus is the phase of the estrous cycle when estradiol levels reach the highest level *(7)*. Thus, the slightly greater vasodilator response observed during proestrus as compared to diestrus may reflect estrogen modulation of the dilatory response. That estrogen may have a significant modulatory role on Ang-(1-7) is consistent with our previous report characterizing Ang-(1-7)'s dilatory response during pregnancy *(4)* and the current observation using estradiol replacement in ovariectomized rats. The importance of estrogen is confirmed by the absence of a vasodilator response both in OVX-PL animals and the virgin animals at DE. The new studies both confirm and extend our original observations by suggesting that estradiol may modulate the magnitude of the Ang-(1-7) vasodilator effect. However, the similar magnitude of the dilator response in pregnancy described in our previous study and with estrogen replacement even though the levels of estradiol were 23-fold higher in our OVX-$E_2$ rats would suggest that estrogen may not be capable of eliciting a dose-dependent effect, but rather that a maximal response to Ang-(1-7) is reached at levels of estradiol found during pregnancy that cannot be further augmented even using pharmacological doses of estradiol. The difference in dilatory responses found during both pregnancy and the estrous cycle may be influenced by the length or magnitude of exposure to estradiol. The estrous cycle in the rat is a 4–5 d cycle with estradiol peaking during proestrus 12 h after diestrus; the proestrus phase has a short duration of 12 h *(7)*. Although we documented that the virgins were in proestrus based on the vaginal smears and the higher level of plasma estradiol as compared to diestrus, the individual estradiol levels showed some variation, suggesting that some animals may have been at the early stage of proestrus and others at the late stage. On the other hand, both pregnant rats that were studied at the 19th d of a 21-d gestation and the estrogen-replaced OVX animals that received 14 d of estradiol had substantially longer duration of exposure. The enhanced dilatory response during pregnancy and with estrogen replacement may be attributed to either the higher levels of estradiol and/or the longer estrogen exposure.

The lack of an Ang-(1-7) dilator response in female Sprague–Dawley (SD) rats at diestrus or when ovariectomized is consistent with our previous report assessing the Ang-(1-7) in vivo depressor response in SD and (m*Ren2*)27 transgenic rats *(3)*. In OVX female SD and (m*Ren2*)27 transgenic rats, Ang-(1-7) did not elicit a depressor response; in contrast in OVX (m*Ren2*)27 transgenic rats with estrogen replacement, but not SD rats, there was a significant depressor response to Ang-(1-7). Because the (m*Ren2*)27 transgenic rats have an extra renin gene and an activated tissue renin–angiotensin system (RAS) *(8)*, the suggestion could be made that an activated RAS may be a condition that can uncover an Ang-(1-7) vasodilator response. This suggestion is supported by studies in pregnancy and low salt diet, two conditions associated with an activated RAS. In pregnant rats there was an augmented dilator response to Ang-(1-7) *(4)* and in rats on a low salt diet there was an augmented contribution of Ang-(1-7) to the maintenance of blood pressure *(9)*. Thus, the status of the RAS, in addition to the reproductive hormonal status, may determine whether an Ang-(1-7)'s dilatory response can be elicited.

In summary, our results suggest that during the normal estrous cycle Ang-(1-7) plays no role in mesentery vasodilation of female SD rats at diestrus, whereas at proestrus a modest dilatory role was observed. Estrogen replacement elicits a significant dilator response to Ang-(1-7) that is mediated by a D-[$Ala^7$]-Ang-(1-7) sensitive site.

## Materials and Methods

### *Animals*

Female SD rats between 10 and 13 wk old were obtained from Charles River Lab (Wilmington, MA, USA). The animals were housed individually for 1–4 wk under a 12-h light/dark cycle in an AAALAC-approved facility. All protocols were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine and are in compliance with NIH guidelines.

### *Surgical Procedure*

Ten-week-old female rats underwent bilateral ovariectomy. The surgery was performed under general anesthesia with ketamine (50 mg/kg im), xylazine (5 mg/kg im), and acepromazine (0.5 mg/kg im). One week after ovariectomy,

pellets containing either 17β-estradiol (5+7.5 mg pellets, 21 d release, Innovative Research of America, Toledo, OH) or placebo were implanted in the subcutaneous tissue of the abdomen. After 2 wk treatment, the rats were sacrificed.

### *Isolated Mesenteric Artery Preparation*

On the day of the experiments a vaginal smear was obtained from the virgin female rats and the vaginal cytology was used to determine the stage of the estrous cycle. All groups of rats were sacrificed by decapitation, and a segment of the proximal jejunum with the mesenteric vasculature attached was excised and placed in ice-cold (4°C) physiological salt solution with the following composition (in mmol/L: KCl 4.8, $CaCl_2$ 2.0, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, dextrose 11, NaCl 118, and $NaHCO_3$ 25). Arteries with an outer diameter of 230–290 μm were identified, carefully dissected, and cleaned of adherent adipose tissue. Isolated artery segments with a length of 2–3 mm were transferred to an arteriograph chamber (Living System Instrumentation, Burlington, VT) *(10)*. The artery segment was cannulated at both ends and maintained at an intraluminal pressure of 40 mmHg. Only leak-free preparations that maintained a stable intraluminal pressure were included. Pre-warmed buffer (37 ± 0.5°C) equilibrated with 21% $O_2$: 5% $CO_2$: 74% $N_2$ (pH 7.4) was circulated through the vessel chamber at a rate of 38 mL/min; the same gas mixture flowed under the superfusion gas cover. The chamber was set on the stage of an inverted microscope with a video camera attached to the viewing tube. The vessel image was projected onto a TV monitor and continuous measurement of the lumen diameter was made using a Living Systems Instrumentation video dimension analyzer system. Signals from a pressure servo system and video dimension analyzer were simultaneously collected by a computer data acquisition system (WinDaq, DATAQ Instruments Inc., Akron, OH), and analyzed by WinDaq Waveform Browser (DATAQ Instruments Inc.). All drugs were added to the circulating buffer reservoir; dosages were expressed as the final molar concentration in the buffer solution. Each mesenteric artery was initially constricted with 50 mmol/L KCl to demonstrate appropriate vascular smooth muscle responses, followed by exposure to $10^{-5}$ *M* acetylcholine to demonstrate intact endothelial-dependent relaxation. Vessels that did not constrict by 50% to KCl and did not dilate by 80%, when acetylcholine was applied, were excluded from further study. After the viability of the vessel had been determined, the vessel was washed and allowed to equilibrate for 30 min prior to the beginning of the experiment. Several arteries were taken from each rat, but only one artery from the same rat was used for each group [i.e. control, Ang-(1-7) or D-Ala/Ang-(1-7)]. At the end of each experiment $10^{-5}$ *M* acetylcholine was added to each vessel to reaffirm the presence of viable endothelium, vessels that did not dilate by at least 80% were excluded from the study.

### *Experimental Protocol*

After an equilibration period, the vessels were preconstricted to approx 30% of their resting diameter with $10^{-7}$ *M* endothelin-1 (ET-1). This dose of endothelin was selected because it does not impair the vasodilator response to Ang-(1-7) *(4,5)*. However, there was some relaxation over time, which necessitated the inclusion of a time control group, but which also allowed for the response to be corrected from the spontaneous relaxation. In a time control group, the luminal diameter of the pre-constricted vessel was recorded for 21 min, i.e., the total time of the experiment. In a second group of vessels, Ang-(1-7), at progressively increasing cumulative concentrations ($10^{-10}$ to $10^{-5}$ *M*), was applied to the ET-1 preconstricted vessel. Vessels were exposed to each peptide concentration for 3 min before the next concentration was added, and the maximal dilation recorded during each 3 min period. In a third group, vessels were perfused with $10^{-7}$ *M* D-Ala$^7$-Ang-(1-7), the specific Ang-(1-7) antagonist, for 30 min during the equilibration period before the vessels were pre-constricted with ET-1 and the concentration–response curve for Ang-(1-7) was obtained. The above experimental protocol was repeated for each animal group, OVX-$E_2$, OVX-PI, and virgin female rats. For each vessel and each peptide, dilation was expressed as the change in luminal diameter from the ET-1 preconstricted state divided by the decrease in baseline diameter evoked by ET-1. The spontaneous relaxation of the time control was subtracted, as previously described *(5)*.

### *17β-Estradiol Measurement*

Plasma 17β-estradiol concentrations were determined by radioimmunoassay with the use of a commercially available kit (Polymedco, Inc.), as previously described *(4)*. The minimum detectable level (MDL) of the assay is <14.8 pmol/L (i.e., <4.7 pg/mL). Measurements that were at the MDL were arbitrarily assigned that value for statistical purposes.

### *Statistical Analysis*

General linear models were used to estimate mean percentage dilation across time while controlling for correlations among measurements and artery segments from the same animal. These models were fit using maximum likelihood; comparisons between means were performed using the Wald test *(11)*. Responses at each concentration of Ang-(1-7) were compared by one-way analysis of variance (ANOVA) followed by Newman–Keuls or Dunn's multiple comparisons test to determine differences between groups. A log transformation was done on the estradiol data to normalize the data, before statistical analysis was done. A $p$ value less than 0.05 was considered statistically significant. Unpaired Student's t-test was used to compare vessel diameter before and after ET-1. All values are presented as mean ± SEM.

### *Reagents and Chemicals*

The chemicals used were from Sigma (St. Louis, MO, USA) unless otherwise noted. Ang-(1-7) and D-[Ala$^7$]-Ang-(1-7) were obtained from Bachem, Inc. (Torrance, CA, USA). These peptides were dissolved in water at an initial concentration of $10^{-2}$ *M*, aliquoted, and stored at –20°C until use. ET-1 was obtained from Novabiochem (La Jolla, CA) and dissolved in 50:50 ethanol:water solution at an initial concentration of $10^{-4}$ *M* and stored at –20°C. On the day of the experiment, the agonists and antagonists were diluted in Krebs-Henseleit solution to achieve the desired final concentration.

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