Endothelium-Dependent and -Independent Mechanisms of Vasorelaxation by Corticotropin-Releasing Factor in Pregnant Rat Uterine Artery

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ABSTRACT
Corticotropin-releasing factor (CRF), a potent vasorelaxant, is increased tremendously during human pregnancy. Placenta is the main source for this increase. CRF is thought to be important in modulating vascular resistance and uteroplacental blood flow during pregnancy. Here we investigated pathways mediating a vasorelaxant effect of CRF in the uterine artery. Two-millimeter segments of uterine artery (o.d. 300–400 μm) from day 18 pregnant rats were mounted in a small vessel myograph and precontracted with norepinephrine, and relaxation responses to CRF were studied. CRF relaxed the uterus artery in a concentration-dependent manner. Relaxation of uterine artery by CRF was abolished completely by α-helical CRF-9-41 (CRF antagonist, 1 μmol) and partially by removal of endothelium, Nω-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor, 0.2 mmol), 6-anilino-5,8-quinolinedione (cytochrome P-450 inhibitors, 0.3 mmol/30 μmol), by indomethacin (cyclo-oxygenase inhibitor, 10 μmol). Relaxation was also inhibited when depolarizing solution (K⁺, 120 mmol) was used for precontraction. In deendothelialized preparations, relaxation was not inhibited by 9-tetrahydro-2-furanyl-9H-purin-6-amine (adenylate cyclase inhibitor, 0.2 mmol), glibenclamide (adenosine triphosphate-dependent K⁺ channel blocker, 1 μmol), tetrabutyl ammonium (nonspecific K⁺ channel blocker, 1 mmol), nitrendipine (voltage-gated Ca²⁺ channel blocker, 1 μmol), or when vessels were precontracted with depolarizing solution. CRF causes vasorelaxation by receptor-operated, endothelium-dependent and -independent pathways. The endothelium-dependent relaxation is mediated by nitric oxide-cyclic guanosine monophosphate pathway and endothelium-derived hyperpolarizing factor but not prostacyclin. However, cyclic adenosine monophosphate, K⁺ channels, or Ca²⁺ channels are not involved in endothelium-independent vasorelaxation by CRF.

Corticotropin-releasing factor (CRF), a 41-amino acid peptide, is produced by the hypothalamus (Rivier et al., 1982). It acts on the anterior pituitary to release adrenocorticotropic releasing factor, which, in turn, stimulates cortisol production by the adrenal glands (Rivier et al., 1982). Central administration of CRF in rats has been shown to elevate blood pressure and heart rate (Fisher et al., 1983). In contrast, peripherally administered CRF lowers blood pressure (Gardner et al., 1988). In addition to control of cortisol levels in the body, CRF may play a direct role in regulation of blood pressure.

In humans, the levels of CRF are normally undetectable in the nonpregnant state, increase exponentially during pregnancy, particularly during the final weeks, and then decline in the immediate postpartum period (Campbell et al., 1987). The placenta is the main source of this increased production of CRF during pregnancy (Riley et al., 1991). CRF levels are higher in pregnancies complicated with pre-eclampsia as compared with uncomplicated pregnancies (Riley et al., 1991). Pregnancy is associated with various cardiovascular changes such as increased blood volume and cardiac output and decreased blood pressure and peripheral vascular resistance (Poston et al., 1995). The decrease in peripheral vascular resistance has been attributed to decreased responsiveness to vasopressor agents and increased production of vasorelaxants. We and others have shown that CRF is a potent vasorelaxant (Lei et al., 1993; Clifton et al., 1995; Jain et al., 1997, 1998). Therefore, CRF may have a role in the modulation of the peripheral vascular resistance during pregnancy, especially of the uteroplacental vascular bed.

The mechanism of the vasodilatory effect of CRF is not well characterized. Vasorelaxants are known to act on the vascular endothelium to cause the release of relaxant factors such as prostacyclin (Moncada and Vane, 1979), nitric oxide (NO) (Furchgott, 1993), and endothelium-derived hyperpolarizing factor.
factor (EDHF) (Vanhoutte, 1996), which, in turn, diffuse to the vascular smooth muscle and cause relaxation. Vasorelaxants can also act directly on the vascular smooth muscle causing an increase in the intracellular cAMP/cGMP levels (Little et al., 1984) or opening of the membrane K+ channels (Brayden and Nelson, 1992), thereby causing relaxation. The vascular endothelium as well as smooth muscle has CRF-binding sites (Dashwood et al., 1987). We have shown previously that the relaxant effect of CRF in rat aorta is predominantly endothelium-dependent and mediated by the NO-cGMP pathway (Jain et al., 1997). Our results agree with those of Clifton et al. (1995), who demonstrated that NO and cGMP are involved in the vasodilatory effect of CRF in the human fetoplacental circulation. However, Lei et al. (1993) showed that in the rat mesenteric artery, vasodilation by CRF is endothelium-independent. Hence, the mechanism of action of CRF remains a subject of controversy.

Because the uterine vasculature may be an important target for CRF during pregnancy, the objective of this study was to examine the mechanism of action of CRF on the uterine artery of pregnant rats. We hypothesized that CRF causes relaxation of the uterine artery by predominantly endothelium-dependent mechanisms.

Materials and Methods

Animals. Timed-pregnant Sprague-Dawley rats (on day 18 of gestation) were obtained from Charles River Laboratories (Wilmington, MA). They were housed separately in temperature- and humidity-controlled quarters with constant light/dark cycles of 12 h:12 h and were provided with food and water ad libitum. The pregnant rats used in these studies have a gestation of 22 days, with day 1 as the day the sperm plug was observed. The animals were sacrificed by CO2 inhalation. Each experimental group consisted of five to eight rats. All procedures were approved by the Animal Care and Use Committee of the University of Texas Medical Branch.

Drugs and Solutions. The drugs used in the experiments were acetylated hydrochloride, CRF, α-helical corticotropin-releasing factor 9–41 (CRF-A), NO-nitro-l-arginine methyl ester (l-NNAME), norepinephrine bitartrate (NE), phentolamine hydrochloride, sodium nitroprusside, thiopental sodium, indomethacin, and tetraethylammonium chloride (TBA) were purchased from Sigma (St. Louis, MO); 6-anilino-5,8-quinolinedione (AQD, LY-83,583; Alexis, San Louis, MO); α,β-unsaturated ketone, 10–4 mol), and pinacidil, and 9-tetrahydro-3-furyl-1H-pyridinum (TFPA, SQ-22,536) were purchased from Research Biochemicals International (Natick, MA). Stock solutions of all of the drugs were prepared in deionized water with the exception of indomethacin (10−2 mol), which was prepared in a 150-mmol solution of NaHCO3 (pH 8.3), and of AQD (10−1 mol), glibenclamide (10−2 mol), and pinacidil (10−1 mol), which were dissolved in dimethyl sulfoxide. Stock solutions for NE were freshly prepared for each experiment. The composition of physiological salt solution was as follows: NaCl, 115 mM; KCl, 5 mM; NaH2PO4, 1.2 mM; NaHCO3, 25 mM; MgCl2, 1.2 mM; CaCl2, 2.5 mM; EDTA, 0.026 mM; and glucose, 11mM. The depolarizing solution (high-K+ physiological salt solution) was made by replacing NaCl with equimolar KCl; the final K+ concentration in that solution was 120 mM.

In Vitro Experiments. Two-millimeter segments of the uterine artery (o.d. 300–400 μm) were mounted in the jaws of a wire myograph (model 410A; J.P. Trading Inc, Aarhus, Denmark) over 25-μm tungsten wires. The preparations were bathed in physiological salt solution maintained at 37°C, pH 7.4. A mixture of 95% O2 and 5% CO2 was bubbled continuously through the solution. The vessels were given a preload based on the length–tension curve for each vessel. Myosight software (J.P. Trading Inc) was used for estimating the circumference that each vessel would have had under a transmural pressure of 100 mm Hg in situ, and the circumference of the preparation was adjusted to 90% of the estimated circumference (Mulvany and Halpern, 1977). The vessels were equilibrated for 1 h. Then, two successive stimulations of 15-min duration were given with high-K+ physiological salt solution, separated by a 15-min equilibration in physiological salt solution. The endothelium was removed in some vessels by rubbing their luminal surface with a human hair (Ossel et al., 1989). The presence or absence of endothelium in the preparations was confirmed by contracting with NE (10−6 mol) and eliciting a relaxation with acetylcholine (10−6 mol). After washing and rest, the preparations with or without endothelium were contracted with NE (10−6 mol) and the relaxant responses to cumulative concentrations of CRF (10−10 to 10−4 mol) were studied. The force was recorded by an isometric force transducer and analyzed using Windaq data acquisition and playback software (DataQ Instruments, Inc., Akron, OH).

Vasorelaxation by CRF was studied in the presence of CRF-A (CRF receptor antagonist, 10−8 mol, with preincubation for 15 min, which was shown to be sufficient in the preliminary experiments) to ascertain whether relaxation by CRF is a receptor-mediated or non-specific effect. To assess the role of endothelium, the responses to CRF were studied in the vessels denuded of endothelium. The relaxation by CRF was also assessed in preparations with intact endothelium preincubated with l-NNAME (NO synthase inhibitor, 10−4 mol, for 30 min) and AQD (soluble guanylate cyclase inhibitor, 10−5 mol, for 30 min) to investigate the role of NO-cGMP pathway in vasorelaxation by CRF. In preliminary experiments, inhibition of NO synthase or guanylate cyclase was confirmed by contracting the preparations with NE and relaxing with acetylcholine (10−6 mol) or sodium nitroprusside (10−7 mol), respectively. The responses to CRF in vessels contracted with depolarizing solution (120 mmol of K+) were compared with those contracted with NE to assess the involvement of EDHF in relaxation by CRF. Responses also were studied in vessels preincubated with cytochrome P-450 inhibitors, thiopental (3×10−4 mol), or miconazole (3×10−5 mol) for 30 min to investigate the role of endothelium in the preparations with intact endothelium preincubated with evitracitin (cyclo-oxygenase inhibitor, 10−5 mol, for 45 min).

To investigate mechanisms responsible for direct effects of CRF on the vascular smooth muscle, responses were examined in denedothelized preparations preincubated with TFPA (adenylate cyclase inhibitor, 2×10−4 mol, for 30 min), glibenclamide (adenosine triphosphate-sensitive K+ channel blocker, 10−5 mol, for 30 min), or TBA (non-specific K+ channel blocker, 10−3 mol, for 15 min). Inhibition of adenylyl cyclase or K+ channels was confirmed by relaxing the preparations with ciprofloxacin (prostacyclin analog, 10−6 mol) or pinacidil (adenosine triphosphate-sensitive K+ channel opener, 10−5 mol), respectively. To assess the role of Ca2+ channels, responses to CRF were studied in denedothelized preparations preincubated with phenolamine (alpha adrenergic receptor blocker, 10−6 mol) and contracted with 120 mmol of K+ (to selectively activate voltage-gated Ca2+ channels) or preincubated with nitrendipine (voltage-gated Ca2+ channels blocker, 10−6 mol, for 30 min) and contracted with NE. Inhibition of voltage-gated Ca2+ channels with nitrendipine was confirmed by contracting preparations with depolarizing solution.

Data Analysis. Data are expressed as mean ± S.E., and n represents the number of rats used in each experiment. The effect of CRF on the uterine artery was quantified as the percentage of relaxation of the preexisting tone in preparations contracted with NE or depolarizing solution. Concentration-response curves were generated based on responses to cumulative concentrations of CRF. The median effective dose (ED50) values for CRF (concentration of CRF producing 50% of the maximal relaxation) were calculated. Area under the dose-response curves was calculated and expressed in arbitrary units. For statistical analysis, Student’s t test or one-
CRF relaxed the uterine artery of day 18 pregnant rats in a concentration-dependent manner (Fig. 1). Blockade of the CRF receptor by CRF-A abolished relaxation of the uterine artery by CRF (Fig. 2). The area under the concentration-response curve was significantly decreased (control, 195.15 ± 7.15; CRF-A, 58.05 ± 11.93, p < .001). Relaxation responses of the uterine artery to CRF were significantly inhibited by removal of the vascular endothelium (ED50: control, 129.42 ± 6.86; deendothelialized, 7.83 ± 0.07, p < .001; area under the curve: control, 129.74 ± 10.26, p < .001), whereas only the decrease in the area under the curve was significant in the case of AQD (ED50: control, 11.49 ± 7.15; area under the curve: control, 142.26 ± 17.83; AQD, 45.45 ± 2.15, p < .001).

When depolarizing solution (120 mmol of K+) was used for contracting the uterine artery, relaxation by CRF was decreased as compared with uterine artery contracted with NE (Fig. 5). In addition, in preparations contracted with NE, preincubation with thiopental or miconazole (inhibitors of cytochrome P-450) significantly decreased relaxation responses to CRF (Fig. 5). The ED50 values were not significantly decreased by 120 mmol of K+, thiopental, or miconazole, but the areas under the curves were decreased significantly (Table 1). Inhibition of cyclo-oxygenase by indomethacin did not affect the relaxation responses to CRF in the uterine artery (Fig. 6). The ED50 value or the area under the curve was not significantly different from controls (ED50: control, −7.73 ± 0.07; indomethacin, −7.63 ± 0.04, p > .05; area under the curve: control, 129.74 ± 13.54; indomethacin, 129.42 ± 10.26, p > .05).

In vessels denuded of endothelium, the relaxation was not decreased by inhibition of adenylyl cyclase with TFPA, inhibition of adenosine triphosphate-sensitive K+ channels with glibenclamide, or nonspecific inhibition of K+ channels with TBA (Fig. 7). The ED50 Values and the areas under the curves were not significantly different (Table 2). In addition, selective activation of voltage-gated Ca2+ channels by depolarizing solution (120 mmol of K+), in presence of phenolamine) or inhibition of voltage-gated Ca2+ channels with nitrendipine did not alter the relaxation responses to CRF (Fig. 8). The ED50 values and the areas under the curves were not significantly different (ED50: NE, −6.80 ± 0.16; 120 mmol of K+, −6.86 ± 0.14; NE/nitrendipine, −7.23 ± 0.20, p > .05; area under the curve: NE, 34.60 ± 13.15; 120 mmol of K+, 27.86 ± 6.90; NE/nitrendipine, 39.60 ± 11.49, p > .05).

### Discussion

CRF is a potent vasodilator that has been shown to cause hypotension when administered i.v. in rats (Gardiner et al., 1988). We have shown previously that CRF, when administered chronically in pregnant rats, causes a decrease in blood pressure (Jain et al., 1998). We also have shown that CRF is a relaxant of the rat aorta in vitro and this relaxant effect is endothelium-dependent and mediated by the NO-cGMP pathway (Jain et al., 1997). The objective of the present study...
was to characterize the effects of CRF on rat uterine artery during pregnancy. Our study demonstrates that CRF is a potent relaxant of the uterine artery of pregnant rats. Vasorelaxation by CRF is a specific, receptor-operated effect. This effect is predominantly endothelium-dependent and mediated by the NO-cGMP pathway as well as EDHF but not prostacyclin. There is also a smaller endothelium-independent component of vasorelaxation by CRF. Neither cAMP, K channels, nor Ca channels are involved in endothelium-independent relaxation by CRF.

CRF has binding sites on the vascular endothelium as well as smooth muscle (Dashwood et al., 1987). Two subtypes of the CRF receptor have been identified in humans and rats: CRF₁ and CRF₂ (Grigoriadis et al., 1996). CRF₂ has two splice variants, i.e., CRF₂a and CRF₂b. The results from a previous study suggest that the CRF receptor in the vasculature may be type 2 (Rohde et al., 1996). Our studies on the CRF receptor indicate that the isoform expressed in the vasculature is CRF₂b (unpublished observations). Hence, our studies support the conclusion that vasorelaxant effect of CRF is mediated by the receptor CRF₂b.

Vascular endothelium is known to be important in the regulation of vascular smooth muscle tone through various contracting (endothelin, angiotensin II) and relaxing factors (NO, prostacyclin, EDHF) (Rubanyi, 1993). In the present study, we show that removal of endothelium abolishes a major component of relaxation of the uterine artery by CRF, especially at lower (and more physiological) concentrations of CRF. This supports the conclusion that vasorelaxant effect of CRF is endothelium-dependent. Release of endothelial relaxing factors is mediated by an increase in free cytoplasmic Ca²⁺ levels in the endothelial cells (Furchgott, 1983). This increase is effected by various mechanisms such as influx of extracellular Ca²⁺, Na⁺–Ca⁺⁺ exchange, and liberation of Ca⁺⁺ from intracellular stores (Singer and Peach, 1982; Winquist et al., 1985; Luckhoff and Busse, 1986). CRF has been shown to cause an increase in intracellular calcium through receptor-operated Ca⁺⁺ channels (Kiang, 1994). Hence, CRF may enhance the production of endothelial relaxing factors by increasing intracellular Ca⁺⁺ in the endothelium. Our studies on localization of the CRF receptor by immunohistochemistry show that the CRF receptor is present predominantly in the endothelium and, to a lesser extent, in the...
vascular smooth muscle (unpublished observations) and therefore support this conclusion.

Endothelium-derived relaxing factor or NO is generated in the vascular endothelium from \( \text{L-arginine} \) by a \( \text{Ca}^{2+} \)-dependent NO synthase (Furchgott, 1993; Wu, 1995). NO activates the soluble guanylate cyclase of the vascular smooth muscle and increases cGMP, which causes relaxation of the smooth muscle (Holzmann, 1982). In the present study, blockade of the NO synthase with L-NAME or of soluble guanylate cyclase with AQD caused a significant inhibition of the relaxant effect of CRF. L-NAME is a prodrug that lacks NO synthase blocking activity and is rapidly hydrolyzed in biological tissues to its active form, i.e., \( \text{N}^\bullet\text{-nitro-L-arginine} \) (Pfeiffer et al., 1996). Similarly, AQD, an inhibitor of soluble guanylate cyclase, can potentiate intracellular release of NO (Kawada et al., 1994). However, in spite of potential nonspecific actions of these agents, the similarity of the effects of de-endothelization, L-NAME, and AQD support this role of endothelium, NO, and cGMP in vasorelaxation by CRF. It may be noted that NO may cause relaxation of smooth muscle by mechanisms other than stimulation of guanylate cyclase (Barany, 1996). Prostacyclin, the first endothelial relaxing factor to be identified, is produced by the cyclo-oxygenase enzyme (Moncada and Vane, 1979). However, inhibition of cyclo-oxygenase with indomethacin failed to have any effect on the vasorelaxation by CRF. This indicates that prostacyclin may not be an important factor that mediates endothelium-dependent relaxation by CRF.

A recently described endothelial factor, EDHF, has been
one class of EDHFs (epoxyeicosatrienoic acids) has been shown to be produced by cytochrome P-450 (Lischke et al., 1996). Inhibition of adenylate cyclase by TFPA (a specific adenylate cyclase inhibitor) (Lippe and Ardizzone, 1991) or blockade of adenosine triphosphate-sensitive K⁺ channels (with glibenclamide) or nonspecific inhibition of K⁺ channels (with TBA) failed to inhibit the direct relaxant effect of CRF in deendothelialized uterine artery. Hence, neither K⁺ channels nor cAMP may mediate the direct relaxant effect of CRF on the vascular smooth muscle. In addition, selective activation of voltage-gated Ca⁺⁺ channels by depolarizing solution in the presence of α-adrenoceptor blocker phenolamine as well as inhibition of voltage-gated Ca⁺⁺ channels by nitrendipine did not decrease relaxation responses to CRF, indicating that Ca⁺⁺ channels may not be directly involved in this effect.

Our data confirm the results from our previous study (Jain et al., 1997) as well as those of Clifton et al. (1995) which show that the relaxant effect of CRF is endothelium-dependent and mediated by the NO-cGMP system. However, our results are only in partial agreement with Lei et al. (1993), who showed that the response to CRF in small mesenteric arteries from male Wistar rats (precontracted with arginine vasopressin) is endothelium-independent. Because CRF exhibits regional differences in its effects (Gardiner et al., 1988), it is possible that the mechanism characterized in our study is different from the one examined in the previous report. Differences in the agonist used to precontract the vessel and the gender and strain of rats used are other factors that may account for the observed differences.

This study shows that CRF is a potent relaxant of the pregnant rat uterine artery. The relaxation responses to CRF in the rat uterine artery as well as aorta are decreased at the term of gestation (Jain et al., 1997, 1998). The nonpregnant rat uterine artery is also relaxed by CRF (unpublished data). In addition, CRF receptor expression in the rat uterine artery is decreased at the end of pregnancy. Thus, this mechanism of relaxation is actively regulated during pregnancy. In humans, CRF production is increased exponentially from low-picomolar concentrations in the nonpregnant state to ~2 nmol toward the end of pregnancy (Campbell et al., 1987). The placenta is the main source for CRF in pregnant women as opposed to the nonpregnant state, when the hypothalamus produces very low amounts of this peptide (Riley et al., 1991). However, the circulating levels of CRF levels were not increased during pregnancy in rats (Jain et al., 1998). Nonetheless, infusion of the CRF antagonist in pregnant rats caused a decrease in blood pressure, supporting the presence of a vasoactive level of CRF bioactivity during pregnancy in rats (Jain et al., 1998). Various other peptides are known to act on the CRF receptor, e.g., urocortin, urotensin I, sauvagine, and epidermal growth factor (Brown et al., 1982; Polk et al., 1987; Vaughan et al., 1995). Urocortin, which is produced by the placenta (Petraglia et al., 1996), has been shown to be more active on the CRF receptor type 2 (Vaughan et al., 1995), suggesting that it may be the true high-affinity ligand for this receptor isomorph. Therefore, it would be reasonable to assume that not only CRF but also other CRF-related peptides that may be increased during pregnancy may act on the CRF receptors and cause vasorelaxation in the uteroplacental bed. The increase in CRF levels in pregnant women is even greater in pregnancies complicated by pre-eclampsia (Campbell et al., 1987). Because pre-eclampsia is associated with uteroplacental hypoperfusion (Sibai, 1996), the abnor-

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**TABLE 2**

Effect of inhibition of adenylate cyclase (TFPA) and K⁺ channels (glibenclamide/TBA) on relaxation by CRF

One-way analysis of variance followed by Newman–Keuls multiple comparisons test was used, and the means were not significantly different; p > .05.

<table>
<thead>
<tr>
<th></th>
<th>ED₅₀ (log mol)</th>
<th>Area Under Curve (arbitrary unit)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>-7.01 ± 0.19</td>
<td>37.18 ± 14.15</td>
</tr>
<tr>
<td>TFPA</td>
<td>-7.06 ± 0.16</td>
<td>33.39 ± 7.87</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>-7.38 ± 0.17</td>
<td>49.23 ± 9.60</td>
</tr>
<tr>
<td>TBA</td>
<td>-7.08 ± 0.13</td>
<td>35.20 ± 3.85</td>
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**Fig. 8.** Effect of selective activation of voltage-gated Ca⁺⁺ channels (120 mmol of K⁺) or inhibition of voltage-gated Ca⁺⁺ channels (nitrendipine) on responses of deendothelialized uterine artery of day 18 pregnant rats to cumulative concentrations of CRF. The vessels were preincubated with phenolamine (10⁻⁶ mol) and contracted with depolarizing solution (120 mmol of K⁺) or preincubated with nitrendipine (10⁻⁶ mol) for 30 min and contracted with NE (10⁻⁷ mol). The 100% contraction induced by NE was 2.02 ± 0.12 g, by depolarizing solution was 1.76 ± 0.14 g, and by NE, nitrendipine was 1.13 ± 0.17. Each point represents mean ± S.E., n = 6. One-way analysis of variance was used; p > .05.
nal increase in CRF in these conditions may represent a compensatory response of the placenta to underperfusion.

In summary, the present study shows that CRF is a potent vasorelaxant that relaxes the uterine artery predominantly through endothelium-dependent but additionally through endothelium-independent mechanisms. This effect may be important in modulating uteroplacental blood flow during pregnancy.

References


