Increased Acetylcholine-Induced Vasodilation in Pregnant Rats
A Role for Gap Junctional Communication

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Abstract—We have tested the hypothesis that increased gap junctional communication contributes to the augmented endothelium-dependent vasodilation in pregnancy. Contractile force and connexin43 expression were measured in aortic rings from nonpregnant and pregnant rats. Norepinephrine-constricted aortas from pregnant rats were more sensitive to acetylcholine, but not to sodium nitroprusside, compared with those from nonpregnant rats. Vessels from pregnant rats, constricted either with 45 mmol/L KCl or with norepinephrine plus $10^{-4}$ mol/L $N^\omega$-monomethyl-l-arginine (L-NMMA), an inhibitor of nitric oxide synthase, also exhibited greater relaxation to acetylcholine. Heptanol, an uncoupler of gap junctional communication, inhibited acetylcholine responses in norepinephrine-constricted aortas from nonpregnant rats but greatly impaired acetylcholine relaxation in aortas from pregnant rats. Heptanol also inhibited in both groups acetylcholine responses in vessels constricted with KCl, only minimally affected acetylcholine relaxation in arteries constricted with norepinephrine plus L-NMMA, and did not change sodium nitroprusside–induced relaxation. Tetraethylammonium chloride induced greater contractions in control aortas compared with aortas from pregnant rats. Increased connexin43 mRNA levels were found in the uterus and in the mesenteric, uterine, and thoracic aortic arteries, but not in the heart and brain, from pregnant rats. These results suggest that increased gap junctional communication, possibly due to increased gap junction protein expression, may facilitate the effects of endothelium-derived relaxing factors, contributing to the augmented endothelium-dependent relaxation in arteries from pregnant rats. (Hypertension. 1999;34[part 2]:937-942.)

Key Words: preeclampsia ■ rats ■ gap junctions ■ nitric oxide ■ endothelium-derived factor

Pregnancy is associated with a decrease in systemic vascular resistance, which maintains or reduces maternal blood pressure despite the increase in plasma volume and cardiac output.1 Pregnancy in women and in other mammals, such as the rat, is also characterized by a blunted pressor response2,3 and decreased vascular reactivity4,5 to a series of vasoconstrictors. The mechanism(s) responsible for these cardiovascular changes in pregnancy is not fully understood but has been under intense investigation, particularly because its malfunction may have a role in preeclampsia.6 Because blunted constrictor responses in arteries from pregnant animals are observed in vitro4,5 and no changes in receptor number or affinity seem to occur,2,7 it has been suggested that a general mechanism, occurring within the blood vessel wall, is involved. Enhanced endothelium-dependent vasodilation, which may contribute to the attenuated vasoconstriction during pregnancy, has been systematically shown in vessels from pregnant animals, including the uterine, mesenteric, thoracic, and abdominal aortic arteries.8–11 Nitric oxide (NO), but not prostaglandins, is considered to contribute substantially to the increased endothelium-dependent relaxation in vessels from pregnant rats, and an increased expression of the endothelial NO synthase enzyme has been described.11 More recently, a role for endothelium-derived hyperpolarizing factor (EDHF) in the increased endothelium-dependent relaxation during pregnancy has also been suggested.8–10

Experimental evidence from both intact tissues and cultured cells supports a role for gap junctional communication (GJC) in the control of arterial function,12–15 and it seems that endothelium-dependent vasodilation, mediated by both NO and EDHF, partly relies on GJC.16–18 Gap junctions are membrane-localized channels that permit intercellular movement of small molecules, facilitating intercellular communication and coordination of cellular activities.13,19 A hexamer of individual connexin proteins constitutes the gap junction, and a family of connexins, having at least 13 identified members in mammals, has been described.13,19 Three of the characterized connexins, connexin43 (Cx43), connexin40...
(Cx40), and connexin37 (Cx37), have been identified in cells of the vascular system, where they may play a role in the control of vasomotor function. Accordingly, the present studies were undertaken to investigate whether increased GJC contributes to the augmented endothelium-dependent vasodilation in pregnancy.

Methods

All of the procedures used were in accordance with guidelines at the University of Sao Paulo, Sao Paulo, Brazil. Age-matched, late-pregnant (20 to 21 days’ gestation) and control nonpregnant Wistar rats were obtained from our breeding stock at the Department of Pharmacology, University of Sao Paulo. Pregnancy was initiated by mating 14-week-old female rats, and the day on which spermatozoa were found in the vaginal smear was labeled day 1 of pregnancy (term, 22 days). Systolic blood pressure was measured by an indirect tail-cuff method (pneumatic transducer, PowerLab 4/S, AD Instruments Pty Ltd).

Isolated-Tissue Experiments

Rats were anesthetized with chloral hydrate (450 mg/kg body weight) and exsanguinated. The thoracic aorta was removed and placed immediately in physiological salt solution (PSS). Vessels were dissected into 4-mm rings, mounted between 2 stainless steel holders, and placed in tissue baths containing PSS at 37°C and aerated with 95% O2 /5% CO2 for isometric force measurements (MLT001 isometric transducers, PowerLab/8S, AD Instruments Pty Ltd). The composition of the PSS was as follows (in mM/L): 130.0 NaCl, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4·7H2O, 14.9 NaHCO3, 1.6 CaCl2, 5.5 dextrose, and 0.03 CaNa2EDTA, pH 7.3. An optimum passive tension (2 g) was applied to the tissues, and they were allowed to equilibrate for 60 minutes in the presence of indomethacin (10−6 mol/L), which was used to eliminate the effects of cyclooxygenase products. In some experiments, the endothelium was removed by gently rubbing the arterial lumen with a cotton swab moistened with PSS. After equilibration, rings were pharmacologically tested for endothelial integrity and challenged with 90 mmol/L KCl. Cumulative concentration-response curves to acetylcholine (ACh, 10−5 to 10−9 mol/L) and sodium nitroprusside (SNP, 10−10 to 10−6 mol/L) were performed in pregnant and nonpregnant rat aortic rings submaximally contracted with norepinephrine (NE). Arteries were stimulated with NE at concentrations (3×10−4 to 3×10−10 mol/L) that allowed similar force generation between both groups. To determine the contribution of NO to ACh-induced relaxation, aortic rings were treated with a selective NO synthase inhibitor, 1N4-monomethyl-L-arginine (L-NMMA, 10−4 to 10−3 mol/L), for 30 minutes before ACh stimulation. To evaluate the contribution of EDHF, ACh stimulation was performed in vessels constricted with KCl (35 to 45 mmol/L). Finally, to evaluate the contribution of GJC to the pregnancy-associated, increased endothelium-dependent relaxation, the protocols described above were performed after previous incubation with heptanol or octanol, reversible inhibitors of GJC (10−3 to 10−4 mol/L, 15 minutes). Responses to tetraethylammonium chloride (TEA, 10−2 to 10−3 mol/L), which promotes gap junctional recruitment, were also evaluated, and each concentration of TEA was equilibrated with the tissues for 20 minutes to allow optimal response.

Identification of Cx43 mRNA in Total RNA Extracts

Total RNA was isolated from the heart, uterus, and arteries (uterine, thoracic aorta, and mesenteric). Tissues were homogenized in TRIzol reagent (Gibco BRL), and after addition of chloroform and centrifugation, RNA was precipitated from the aqueous phase by the addition of isopropanol, washed with ethanol, and solubilized. RNA was quantitated by absorbance measurements at 260 and 280 nm (Hitachi U-1100) with 1 optical density unit considered equal to 40 μg/mL. Integrity of RNA was analyzed by ethidium bromide staining. RNA samples (10 μg per lane) were separated by electro-

Results

On the day of the experiment, the systolic blood pressure of pregnant and nonpregnant rats was 103±2.6 mm Hg, n=24, and 121±0.6 mm Hg, n=22 (P<0.05), respectively. The magnitude of contraction to NE or KCl was not different between arteries from both groups. The Table summarizes values for pD2 and maximal responses.

ACh-Induced Relaxation: Contribution of NO and EDHF

Sensitivity to ACh, expressed by the pD2 values, was greater in NE-constricted arteries from pregnant compared with nonpregnant rats (Figure 1A). Indomethacin (10−5 mol/L) treatment did not change the sensitivity or maximal relaxation to ACh, and all subsequent experiments were performed in the presence of this inhibitor. No differences in SNP response were observed between vessels from pregnant and nonpregnant rats. When the ACh response was evaluated in arteries constricted with KCl, sensitivity and maximal relaxation were significantly greater in arteries from pregnant compared with nonpregnant rats (Figure 1B). In vessels treated with L-NMMA (10−4 mol/L) and constricted with NE, a decreased sensitivity to ACh, expressed by a rightward shift in the ACh curves, and also a significant inhibition of maximal relaxation were observed in both groups (Figure 1C). Arteries from pregnant rats still were more responsive to ACh than were control arteries. In vessels from both pregnant and nonpreg-
nontant rats, ACh-induced vasodilation was completely inhibited when vessels were treated with L-NMMA (10^{-4} mol/L) and constricted with KCl.

### Influence of Heptanol on ACh-Induced Relaxation

Heptanol 10^{-3} mol/L produced a greater inhibition of ACh responses in NE-constricted vessels from pregnant compared with nonpregnant rats (Figure 2). However, NE-induced contraction was also affected by this concentration of heptanol (vehicle=2.6±0.2 g pregnant versus 2.8±0.2 g nonpregnant; heptanol=1.9±0.6 g pregnant versus 1.7±0.3 g nonpregnant). Heptanol, 3×10^{-4} mol/L, a dose that does not affect NE response (vehicle=2.0±0.2 g pregnant versus 2.2±0.4 g nonpregnant; heptanol=2.3±0.2 g pregnant versus 2.0±0.2 g nonpregnant), produced a rightward shift of ACh relaxation in aortas from pregnant rats (Figure 2A) but minimally affected ACh response in vessels from nonpregnant animals (Figure 2B). In this condition, the increased ACh-induced vasodilatation in arteries from pregnant rats was not observed. Heptanol 10^{-4} mol/L produced no significant changes in the ACh response in arteries of both groups. Similar results were obtained when octanol, an 8-chain linear alcohol that also inhibits GJC, was tested on ACh response. ACh-induced relaxation in KCl-constricted arteries from both pregnant and nonpregnant rats was significantly inhibited by heptanol (3×10^{-4} and 10^{-3} mol/L; Figures 3C and 3D). HEptanol minimally affected ACh responses in pregnant and nonpregnant rat aortas constricted with NE after treatment with L-NMMA (Figure 3A and 3B). SNP-induced relaxation in both groups was not significantly changed by 10^{-3} mol/L heptanol. TEA induced greater contraction in control arteries, both in endothelium-intact and endothelium-denuded aortas, compared with arteries from pregnant rats (Figure 4).

Northern blot analysis for Cx43 mRNA was performed to determine whether Cx43 expression was altered in vessels from pregnant rats in comparison with arteries from nonpregnant rats. Cx43 mRNA was detected in all vascular extracts, and increased expression of Cx43 was found in the uterus and in the uterine, mesenteric, and thoracic aortic arteries (P<0.05) but not in the heart or brain from pregnant rats (Figure 5).

### Discussion

The aim of the present study was to evaluate the potential role of GJC on the increased endothelium-dependent vasodilation during pregnancy. The rationale was the observation that relaxation to endothelium-dependent factors, mediated by both NO and EDHF, partially relies on GJC, and both factors seem to play a role in the increased endothelium-dependent vasodilation observed during pregnancy. Using a pharmacological treatment to distinguish between the components of ACh-induced vasodilatation, we have observed that vessels from pregnant rats displayed increased relaxation mediated by both EDHF (ACh response evaluated in the presence of indomethacin and L-NMMA) and NO (ACh response in the presence of indomethacin and KCl). We observed no differences in SNP-induced relaxation, which agrees with previous studies showing no changes in the sensitivity to NO donors between arteries from pregnant and nonpregnant rats. Our results are consistent with other reports describing that EDHF may be increased in vascular segments during pregnancy. Because ACh-induced maximum relaxation was greater in KCl-constricted arteries from the pregnant rats, it seems that enhanced synthesis/efficacy of EDHF per se does not explain the increased ACh vasodilation and that NO or other factors may also play a role in this response. Bobadilla et al. have shown that clotrimazole, an agent that inhibits cytochrome P450 enzymes, decreased maximum ACh-induced relaxation in aortic rings from pregnant rats but did not change ACh responses in aortas from
nonpregnant rats and have suggested the involvement of a P450 metabolite, possibly EDHF, in the increased endothelium-dependent vasodilation in pregnancy. However, other authors have shown that clotrimazole and related imidazole antimycotic agents, besides inhibiting P450 enzymes, may nonspecifically inhibit potassium channels and influence a later component of ACh-induced relaxation.26

Recent experimental evidence has suggested that by using different gap junction inhibitors, heterocellular GJC may account for endothelium-dependent vasodilation, mediated by both NO and EDHF and induced by receptor-dependent and -independent mechanisms.16–18 Gap junction channels provide a direct low-resistance and size-selective pathway for electric and metabolic communication between either homologous or heterologous adjacent cells in most tissues.13,19 Large macromolecules, such as complex lipids, polysaccharides, and proteins, would be unable to cross the gap junction

Figure 1. Line graphs show endothelium-dependent relaxation induced by ACh (10⁻⁹ to 10⁻⁵ mol/L) in aortic rings from pregnant (●) and nonpregnant (●) rats. A, Contraction evoked by NE (1 to 3×10⁻⁷ mol/L); B, contraction by 30 to 45 mmol/L KCl; and C, contraction by NE after 30-minute treatment with L-NMMA (10⁻⁴ mol/L). Experiments were performed in the presence of 10⁻⁵ mol/L indomethacin. Data are expressed as percentage of relaxation of contraction evoked by NE or KCl. Each point represents mean ± SEM of 8 to 10 experiments. *P<0.05 vs nonpregnant rats.

Figure 2. Line graphs show relaxation induced by ACh (10⁻⁹ to 10⁻⁵ mol/L) in thoracic aortic rings constricted with NE (1 to 3×10⁻⁷ mol/L) from pregnant (A) and nonpregnant (B) rats. Arteries were treated with vehicle (ethanol ■, □, 3×10⁻⁴ mol/L heptanol (▲, △), or 10⁻³ mol/L heptanol (●, ○). Experiments were performed in the presence of 10⁻⁵ mol/L indomethacin. Data are expressed as percentage of relaxation of contraction evoked by NE or KCl. Each point represents mean ± SEM of 8 to 10 experiments. *P<0.05 vs ethanol-treated arteries.

Figure 3. Line graphs show relaxation induced by ACh (10⁻⁹ to 10⁻⁵ mol/L) in aortic rings constricted with NE (1 to 3×10⁻⁷ mol/L) after 30-minute treatment with 10⁻⁴ mol/L L-NMMA (top) or 35 to 45 mmol/L KCl (bottom). Arteries from pregnant (A, C) and nonpregnant (B, D) rats were treated with vehicle (ethanol ■, □), 3×10⁻⁴ mol/L heptanol (▲, △), or 10⁻³ mol/L heptanol (●, ○). Experiments were performed in the presence of 10⁻⁵ mol/L indomethacin. Data are expressed as percentage of relaxation of contraction evoked by NE or KCl. Each point represents mean ± SEM of 8 to 10 experiments. *P<0.05 vs ethanol-treated arteries.

Figure 4. Representative tracing (A) and line graphs (B, C) showing contraction induced by TEA (10⁻⁴ to 10⁻¹ mol/L) in thoracic aortic rings from pregnant (P ●, ♦) and nonpregnant (NP ■, □) rats, with (B) or without (C) endothelium. Experiments were performed in the presence of 10⁻⁵ mol/L indomethacin. Data are expressed as percentage of contraction evoked by 90 mmol/L KCl. Each point represents mean ± SEM of 8 experiments. *P<0.05 vs nonpregnant rats.
Heptanol inhibited ACh vasodilation but affected more the NO-dependent than the NO-independent component of the ACh response. Considering that GJC is important for the effects of NO, dependency of the NO pathway on GJC should exist at a stage in that pathway before the NO effects on smooth muscle cells, since SNP relaxation was not altered by heptanol. Increased GJC may be occurring between endothelial–smooth muscle cells and not between smooth muscle–smooth muscle cells. It is difficult to accept the notion that the NO molecule itself travels through gap junctions, since it is a highly diffusible molecule through cell membranes. However, Christ et al. have demonstrated that NO does not have an effective diffusion radius in poorly innervated tissues such as the aorta, and consequently, that diffusion cannot fully account for the NO-induced relaxation. Furthermore, the existence of functional gap junctions in both smooth muscle and endothelial tissue and also between endothelial and smooth muscle has been reported. We can rule out that inhibition of GJC decreases ACh relaxation by interfering with vasodilator prostanooids, because the tissues were treated with indomethacin. A role for GJC in EDHF effects seems to be small in the thoracic aorta, since heptanol had no effects on the ACh response in control arteries treated with L-NMMA. In aortas from pregnant rats, minor inhibition of NO-independent relaxation by heptanol was observed. This may be due to the increased GJC in arteries during pregnancy, which would facilitate EDHF diffusion from the endothelium to smooth muscle cells. In other species, however, GJC seems to contribute to an NO-independent, possibly EDHF, component of endothelium-dependent relaxation. Heptanol may inhibit endothelial gap junctions in arteries from pregnant rats to a greater extent than it does in control arteries, owing to an increased number of gap junction channels in vessels from pregnant animals. Our Northern blot analysis supports this hypothesis, since we observed increased Cx43 mRNA expression in arteries from pregnant rats.

Confirmation that the smooth muscle response to NO is independent of GJC was obtained in aortic rings in which the highest dose of heptanol (10^{-7} mol/L) did not affect relaxation to SNP. Our results differ from those of Javid et al., who observed that both octanol and heptanol inhibited relaxation induced not only by ACh but also by the NO donor S-nitroso-N-acetylpenicillamine. However, our findings support the studies of Chaytor et al., who described that Gap 27, an inhibitory gap junction peptide, inhibited ACh-induced relaxation but did not significantly interfere with SNP response.

The effects of TEA on vascular reactivity of both groups were also evaluated. TEA is used in electrophysiological experiments not only as a tool to inhibit potassium conductance but also as an agent that increases gap junction number and plaque size in nonvascular and vascular smooth muscle cells. TEA also increases Lucifer yellow dye transfer, which has been used as a measure of cell-to-cell communication via gap junctions between vascular smooth muscle cells. The contractions elicited by TEA were greater in arteries from nonpregnant than pregnant rats. One may argue

**Figure 5.** Quantitative analysis of Cx43 mRNA expression in tissues from pregnant rats. After RNA separation and sequential hybridization, densitometric analysis of autoradiograms was performed, and the signal density of each RNA sample hybridized to Cx43 was divided by that hybridized to β-actin. The corrected density for each tissue from pregnant rats is presented as percent increases of values obtained in tissues from nonpregnant rats. The values shown are mean±SEM of data from 3 or 4 separate experiments. *P<0.05 vs nonpregnant rats.

channel, whereas ions and small regulatory molecules would freely cross the membrane. Programmed or physiological modulation of GJC can provide the organism the means to control ionic and small-molecular-weight signal within tissues, since increasing or decreasing gap junctional intercommunication allow cells to act as a source for regulatory signals. A role for gap junctions in vascular physiology has been suggested by different findings: (1) the abundance of mRNA for connexins and presence of the proteins in endothelial and smooth muscle cells from various vascular beds; (2) effective cellular coupling mediated by gap junctions in vascular smooth muscle and endothelial cells, a type of evidence provided by dye transfer techniques; and (3) GJC contributions to vasomotor tone control, a role uncovered by studies on vascular contractility.

We evaluated the effects of heptanol on the ACh response in arteries from control and pregnant rats, after assuming that this gap junction inhibitor effectively inhibits GJC at the doses we used and that its effects are rather selective. The evidence for specific and selective effects of heptanol is that only alkanols of restricted chain length (C6 to C9) are effective in inhibiting GJC. In addition, heptanol at concentrations <2 mmol/L does not alter calcium homeostasis or mobilization, does not affect potassium or chloride channels, and does not change basal tissue tone during incubation. It must be considered that heptanol has other pharmacological effects apart from gap junction inhibition, and we cannot exclude the possibility that the documented anesthetic effects of heptanol may also play a role in the inhibition of ACh responses. However, studies that have compared the effects of heptanol and other inhibitors on GJC, such as sucrose and Gap 27, have found different effects only when very high doses of heptanol were used. Regarding the uncoupling effect of heptanol, it seems to be due to a decrease in the probability of the open state of the gap junctional channel and possibly to a decrease in the fluidity of cholesterol-rich domains of the plasma membrane.
that if arterial GJC is increased in pregnancy, the effects of TEA should be greater in vessels from pregnant rats. However, if one considers that during pregnancy there is an increase in vasodilator factors and that they can freely move through gap junction channels, an increase in GJC would make contractions elicited by TEA more difficult to occur. Furthermore, the differences in TEA-induced contractions between the pregnant and nonpregnant groups were greater in arteries where the endothelium was left intact, a condition in which there is greater availability of vasodilator factors. An increase in myometrial Cx43 protein occurs on days 21 to 22 of pregnancy, before the onset of labor and during delivery in the rat. In myometrial cells, gap junction expression is closely correlated with changes in plasma progesterone/estradiol levels, with the number of gap junctions increasing with increasing estrogen and decreasing progesterone levels. If these hormones indeed modulate gap junction expression and function in the myometrium, it is then possible that they also modulate the expression of Cx proteins in vascular tissue. Gap junctions during pregnancy could act to maintain metabolic and motor coupling of vascular cells to coordinate responses to endocrine signals. An increase in GJC may act to increase the flow of vasodilator factors and consequently, vasodilation during pregnancy.

In summary, the present experiments have shown that in pregnant rats, both NO-dependent and -independent components of ACh vasodilation partially rely on GJC and that Cx43 mRNA expression is increased in arteries from these animals. Collectively, these findings support the hypothesis that GJC is important for vascular reactivity and that intercellular communication through gap junctions may be increased in vessels from pregnant rats.

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