Evidence for Attenuation of Myo-inositol Uptake, Phosphoinositide Turnover and Inositol Phosphate Production in Aortic Vasculature of Rats During Pregnancy

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Abstract

We postulated that vascular phosphoinositide metabolism is attenuated during pregnancy, and thereby could contribute to maternal vasodilation and reduced vascular reactivity. The basal rate of incorporation of [³H]myo-inositol and [³H]glycerol into phosphoinositides of aortae from pregnant rats in vitro was significantly reduced, when compared with vessels from virgin animals. After injection of [³H]myo-inositol intravenously into chronically instrumented conscious pregnant and virgin rats, the incorporation of the label by phosphatidylinositol was $66\pm4\%$ less in aortae of gravid versus virgin animals ($P < 0.001$), despite comparable plasma concentrations of radioactivity. Fold stimulation of total [³H]inositol phosphates by arginine vasopressin, norepinephrine, and angiotensin II over a 15-min period was not different between aortic segments from virgin and gravid rats, although both absolute basal and stimulated levels were significantly less in vessels from pregnant animals. After 45 s of incubation with 10^{-7} M arginine vasopressin, however, the fold-stimulation of [³H]inositol tris-plus tetrakisphosphate was reduced in aortae from gravid rats, when compared with vessels from virgin animals ($P < 0.005$). By HPLC, $> 90\%$ of the radioactivity in the [³H]inositol tris-plus tetrakisphosphate column fraction after 30 and 60 s of agonist stimulation was [³H]inositol-1,4,5-trisphosphate. We further observed that the rate of uptake of [³H]myo-inositol by aortic vasculature obtained from gravid rats was significantly (24%) less than uptake by vessels from virgin animals. Plasma myo-inositol concentrations were not significantly different, but presumably as a consequence of reduced uptake, aortic segments freshly isolated from pregnant rats contained $22\pm6\%$ less myo-inositol than vessels from virgin controls as measured by gas chromatography-mass spectrometry ($P < 0.03$). We conclude that myo-inositol uptake and content, phosphoinositide turnover, and inositol phosphate production are reduced in aortic vasculature of gravid rats. (*J. Clin. Invest.* 1991. 87:1700–1709.) Key words: vasodilation • vascular reactivity • norepinephrine • arginine vasopressin • angiotensin II • chronically instrumented conscious rats

Introduction

Vasodilation of the maternal circulation is a constant feature of normal human pregnancy. Cardiac output, renal blood flow, and glomerular filtration rate increase by 30 to 60% (1–3) while blood pressure declines (4, 5). Furthermore, pressor response to administration of angiotensin II (6) and vascular reactivity to infusion of norepinephrine (7) become attenuated. Insight into the mechanisms responsible for these phenomena may be particularly critical because attenuation of vascular responses to angiotensin II and norepinephrine as well as systemic and renal vasodilation are lost in women who develop preeclampsia (6–9).

We have found that the chronically instrumented, conscious rat manifests alterations in renal and cardiovascular function during gestation, which are remarkably similar to those observed in normal gravid women (10–13). Thus, the pregnant rat may be a useful animal model in which to investigate mechanism(s) responsible for maternal vasodilation and attenuated vascular reactivity to vasoconstrictor hormones. Since blood vessels of gravid rats (14–20), rabbits (21), cats (21), and guinea pigs (22) demonstrate decreased vascular reactivity in vitro or in situ, it seems likely that alterations within the vasculature itself are important. Vasoconstrictor receptor desensitization during pregnancy does not appear to account for the refactory vascular response, at least to angiotensin II and arginine vasopressin (23–26). (The density and affinity of alpha₁-adrenergic receptors on vascular smooth muscle have not been evaluated during pregnancy.) Although controversial, vasodilatory prostaglandins may not be involved in the vasodilation and attenuated vascular reactivity of pregnancy either (11, 22, 27). A possible role for cyclic guanosine-3′,5′-monophosphate and endothelium-derived relaxing factor has been recently suggested (12, 28, 29). Alternatively, we postulated that intracellular processes which mediate vasoconstriction may be altered during gestation such that vasodilation and reduced vascular reactivity occur. As a first step, we examined phosphoinositide turnover, an early event in the signal transducing pathway, which is critical to vascular smooth muscle contraction (30, 31). The results support the view that phosphoinositide turnover, inositol phosphate production, and myo-inositol uptake are significantly attenuated in aortic vasculature of gravid rats. Similar changes in resistance vessels may contribute to vasodilation or to reduced vascular reactivity during pregnancy.

Methods

Long-Evans female rats of ∼ 98 d of age were obtained from Charles River Breeding Laboratories (North Wilmington, MA). They were mated, and day 0 of gestation was documented by the presence of spermatozoa in the vaginal lavage. (Rat gestation lasts 22 d.) Age-matched virgin animals served as controls. Because we used biochemical procedures that involve short term labeling with the radioactive precursor, [³H]myo-inositol and [³H]glycerol, freshly isolated aortic tis-

sue was used in order to obtain sufficient mass of vasculature to yield detectable signals. On the day of study, a gravid (gestational day 18–21) and virgin rat were killed, and the aortae removed and placed in medium (37°C, pH 7.4) preequilibrated with 95% O_2/5% CO_2. After removal of fat and branching vessels, the aortae were gently perfused to remove residual blood. They were then cut into segments with a sharp razor blade. The endothelium was not removed. The isolation procedure lasted no more than 10 min.

Total water-soluble [³H]inositol phosphates. Aortic segments of 0.5–1.0 cm in length were incubated at 37°C in 1.5 ml Earle's Balanced Salt Solution (EBSS)[1] + 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, which contained 6 μCi/ml [³H]myo-inositol (15 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO). The medium was saturated with 95% O_2/5% CO_2 and fresh atmosphere was introduced into the vials every 30 min. After incubation for 1, 2, 3, or 4 h, the tissues were washed with 2.0 ml ice-cold medium (×5). Next, the aortic segments were incubated for 10 min at 37°C in EBSS + 25 mM Hepes, pH 7.4, which contained 10 mM LiCl. LiCl was used to inhibit breakdown of inositol phosphate(s), thus allowing for their accumulation (mainly inositol monophosphate) in order to facilitate detection by scintillation counting. Agonist (norepinephrine, angiotensin II, arginine vasopressin) or vehicle (0.9% saline or medium) was added for a further 15-min incubation. Tissues were homogenized in 1.0 ml 0.6 N $HClO_4$ (4°C). The homogenates were centrifuged at 3,200 g for 10 min and the supernatant neutralized with ~ 120 μl 5.0 N KOH. After another centrifugation step to pellet the $KClO_4$, the supernatant was added to 9.0 ml 5 mM disodium tetraborate. The samples were applied to anion exchange columns (32) (Dowex AG1-X8; Bio-Rad, Richmond, CA). Myo-inositol and glycerophosphoinositol were eluted with two to three successive applications of 10–12 ml each of 60 mM sodium formate/5 mM disodium tetraborate. Total water-soluble [³H]inositol phosphates (mainly inositol monophosphate) were eluted at once with 12 ml 1.0 M ammonium formate/0.1 M formic acid. Duplicate 500-microliter aliquots were counted in 10 ml scintillation fluid (formula 963; New England Nuclear, Boston, MA) for 20 min each (model LS 3801; Beckman Instruments, Inc., Wakefield, MA). In preliminary experiments (n = 3), the 60 mM sodium formate/5 mM disodium tetraborate effectively removed [³H]myo-inositol that failed to come through in the sample void. This solution, however, did not elute [³H]inositol-4-phosphate (< 0.3%), [³H]inositol-1,4-bisphosphate (< 0.14%), or [³H]inositol-1,4,5-trisphosphate (< 0.14%). 1.0 M ammonium formate/0.1 M formic acid eluted 98.7% of [³H]inositol-1,4,5-trisphosphate applied to the columns.

Separation of individual water-soluble [³H]inositol phosphates. Aortic segments of 0.5–1.0 cm in length were incubated at 37°C in 1.5 ml EBSS + 20 mM Hepes, pH 7.4, which contained 10 μCi/ml [³H]-myo-inositol. The medium was saturated with 95% O_2/5% CO_2 and fresh atmosphere was introduced into the vials every 30 min. After incubation for 3 h, the tissue was washed with 1.5 ml ice-cold medium (×5). Aortic segments were then transferred to fresh medium at 37°C (without LiCl), and vehicle or 10^{-7} M arginine vasopressin was added for the indicated times (up to 2 min). Tissues were homogenized in 1.0 ml 0.6 N $HClO_4$ (4°C) which contained phytic acid hydrolysate (~ 26 μg phosphorus) (33). The homogenates were centrifuged at 3,200 g for 10 min and the supernatant was neutralized with 1.25 ml freon/tri-n-octylamine (1:1 vol/vol). After vortexing each sample for 1 min, the phases were separated by centrifugation at 2,000 g for 5 min. The upper phase was then transferred to a graduated tube, and distilled water was added to produce a final volume of 4 ml.

Individual water soluble inositol phosphates were separated by Accell QMA Sep-Pak column chromatography (Waters Associates, Milford, MA) (adapted and modified from reference 33). After application of the sample to the Sep-Pak column, the following reagents were pushed through (10 ml/min): distilled water (5 ml × 3), 5 mM $Na_2B_4O_7$ (5 ml × 3), 0.05 M ammonium formate/0.005 M formic acid (5 ml

× 2), 0.1 M ammonium formate/0.01 M formic acid (5 ml × 4), 0.4 M ammonium formate/0.04 M formic acid (3 ml × 4), and 1.0 M ammonium formate/0.1 M formic acid (5 ml × 4). All solutions, which contained ammonium formate, were made by appropriately diluting the stock solution, 1.0 M ammonium formate/0.1 M formic acid, pH 4.75, with distilled water. Duplicate 500-microliter samples from each fraction were counted by liquid scintillation.

Validation studies using [³H] standards without (n = 3) and with (n = 1 to 5) tissue were performed. The elution profiles were considerably different from those published (33). For [³H]myo-inositol, 99.9% of the standard was found in the void volume and in the initial water washes; for [³H]inositol-4-monophosphate, 98.3% was eluted in the 0.05 M ammonium formate fraction; for [³H]inositol-1,4-bisphosphate, 95.6% was eluted by 0.1 M ammonium formate; [³H]inositol-1,3,4-trisphosphate, [³H]inositol-1,4,5-trisphosphate, and [³H]inositol-1,3,4,5-tetrakisphosphate were all eluted by 0.4 M ammonium formate, 97.5%, 97.5% and 98.7%, respectively. Comparable results were obtained with tissue except that inexplicably, 12.2% of the [³H]inositol-4-monophosphate came out early in the $Na_2B_4O_7$ wash. All radioactive standards were obtained from New England Nuclear, except for [³H]-myo-inositol which was purchased from American Radiolabeled Chemical, Inc.

HPLC was used to resolve the [³H]inositol phosphate (InsP)$_{1,3,4}$ and [³H]InsP$_{1,4,5}$ isomers as well as [³H]InsP$_{1,3,4,5}$ in a few of the 0.4 M ammonium formate fractions from the Sep-Pak columns (33). A Rainin Dynamax HPLC (Rainin Instrument Co. Inc., Woburn, MA) with dual pumps and a Partisil 10 μ SAX column was used. The injectate volume was 1.5–2.2 ml. The mobile phase consisted of a stock solution of 2 M ammonium formate/0.1 M formic acid adjusted to pH 3.7 with orthophosphoric acid. The mobile phase was run at 1.25 ml/min at a final concentration of 0.85 M ammonium formate for the initial 18 min and then 1.80 M from 20 to 30 min. Collection of 18-s fractions were made and counted by liquid scintillation. [³H]InsP$_{1,3,4}$, [³H]InsP$_{1,4,5}$, and [³H]InsP$_{1,3,4,5}$ had retention times of 5.0–6.3, 6.5–7.5, and 20.0–21.8 min, respectively.

Phosphoinositides, in vitro labeling. Aortic segments of 1.0 cm in length were incubated in 6 μCi/ml [³H]myo-inositol (0.47 μM) for 1, 2, 3, or 4 h as described above. They were then washed five times with 2.0 ml ice-cold medium, and homogenized in 2.0 ml chilled $CHCl_3$:CH_3OH: concentrated HCl (200:100:1) and 0.4 ml distilled water. After centrifugation at 1325 g for 5 min to separate the phases, the lower chloroform layer which contained lipids was transferred to a 5.0-ml conical vial (Wheaton Instruments, Millville, NJ). The protein was extracted again with fresh solvent. The lower phase was then combined with the first and stored under N_2 at −40°C. The following day, after removal of a residual aqueous layer, the organic phase was dried (Savant Speedvac Concentrator; Savant Instruments, Inc., Farmingdale, NY or N-Evap; Organomation, South Berlin, MA). Unlabeled phospholipid standards were added to the residue: 25 μg phosphatidylinositol-4,5-bisphosphate, 15 μg lysophosphatidylinositol, 10 μg phosphatidylinositol-4-phosphate, and 10 μg phosphatidylinositol (Sigma Chemical Co., St. Louis, MO). The sides of the vials were washed with an additional 0.5 ml of the acidified extraction mixture, and after drying, the residue was resuspended in 100 μl $CHCl_3$:CH_3OH:H_2O-37.5:12.5:1, and sonicated for 5 min (Branson Co., Shelton, CT). Thin layer plates coated with Silica Gel H + 1% potassium oxalate (Analtec Inc., Newark, DE) were activated for 30 min at 110°C. Samples were applied in 1.0-cm lanes, and 50 μl $CHCl_3$:CH_3OH:H_2O was again added to each vial, and then applied to the TLC plates. Lipids were separated by a moving phase of $CHCl_3$:CH_3OH:4 M NH_4OH 45:35:10 (34). The spots were visualized with iodine vapor, and after sublimation, scraped into 10 ml scintillation fluid (formula 963; New England Nuclear) and each counted for 20 min.

In several experiments, tissues were labeled with [³H]myo-inositol in medium which contained concentrations of myo-inositol (Sigma Chemical Co.) within the physiological range. That is, unlabeled myo-inositol was added to the incubation medium to achieve a final concentration of 50 μM. In some experiments Ham's F12:Eagles Minimum Essential Medium (1:1; Hazelton Systems, Inc., Denver, PA) was used

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1. *Abbreviations used in this paper:* EBSS, Earl's balanced salt solution; InsP, inositol phosphate; MI, myo-inositol.

instead of EBSS. The myo-inositol concentration of this mixture was 55 μM.

Additional experiments were conducted using [³H]glycerol as a precursor (New England Nuclear). Aortic segments of 1.0 cm length were incubated in EBSS + 20 mM Hepes, saturated with 95% O_2/5% CO_2, pH 7.4, which contained 50 μM myo-inositol (MI), 100 μM glycerol, and 6 μCi/ml [³H]glycerol. Three aortic segments were used from each animal. One of the segments was incubated for 1.5 h, a second segment for 3.0 h, and a third also for 3.0 h. The third segment, however, was also incubated with 1 μM norepinephrine during the last 1.5 h of incubation. The tissues were then thoroughly washed in ice-cold medium (×5). Phospholipids were extracted and separated by TLC, and counted as described above. Because phosphatidylinositol and phosphatidylserine comigrate in the TLC system previously described, they were scraped from the plate, extracted from the gel, applied to another TLC plate, and separated by a moving phase of $CHCl_3$:CH_3OH:CH_3-$COOH$:H_2O (81:10:45:1) (35).

Phosphoinositides, in vivo labeling. Midpregnant and age-matched virgin rats were chronically instrumented with a femoral venous catheter using aseptic technique (11). 5–7 d later, the animals were administered [³H]myo-inositol intravenously in a total volume of 250 μl 0.9% saline. Gravid and virgin rats received 43 μCi/275 g and 50 μCi/275 g body weight, respectively. The catheter was then flushed with an additional 1.0 ml of 0.9% saline. Blood ($\sim$ 50 μl) was drawn at 5, 15, 30, 60, and 120 min after the injection of [³H]myo-inositol, and after centrifugation the plasma was counted for radioactivity (10 μl in duplicate). At 120 min, the rats were administered 40 mg/kg i.v. pentobarbital. A laparotomy was quickly performed. The animal was killed by rapidly withdrawing 10–20 cc of blood from the abdominal aorta. The aorta was then removed and placed in ice-cold medium. It was next cleaned, perfused, and cut into two 2.0-cm segments. These segments were thoroughly washed in ice-cold medium (×5), subjected to organic extraction, and the phospholipids separated by TLC and counted as described above.

Myo-inositol uptake. Aortic segments were preincubated for 1 h in EBSS + 20 mM Hepes, saturated with 95% O_2/5% CO_2, pH 7.4, in the presence and absence of K^+. To maintain constant osmolality, additional NaCl was added to the K^+-free medium. The tissues were then transferred to fresh medium, again with and without K^+, which contained 1 μCi/ml [³H]myo-inositol and 0.25 μCi/ml [¹⁴C]mannitol (American Radiolabeled Chemical, Inc.). Unlabeled myo-inositol was added to produce a final concentration of 35, 50, or 70 μM. After incubations for 2, 5, 10, 20, 60, or 120 min, the tissues were vigorously, but briefly washed in ice-cold medium and then homogenized in 1.0 ml 0.6 N $HClO_4$. After centrifugation at 3,200 g for 10 min, the supernatants were neutralized with $\sim$ 140 μl 5.0 N KOH, and the $KClO_4$ was removed by an additional centrifugation step. Radioactivity of the supernatant was determined for [³H] and [¹⁴C]. Corrections for efficiency and channel crossovers were made. Extracellular [³H]myo-inositol was subtracted from total to obtain intracellular [³H]myo-inositol. In an additional experiment, 1 mM final concentration of ouabain was used, instead of deleting K^+ from the medium.

Plasma concentration of myoinositol, enzymatic-fluorometric assay (adapted and modified from references 36 and 37). 500 μl of plasma was chilled on ice and treated with 500 μl of ice-cold 0.6 N $HClO_4$ for 15 min. The mixture was vortexed every 5 min and the precipitated proteins were pelleted by centrifugation. The supernatant was then treated with 5.0 N KOH until the pH was 7.0. After another centrifugation step, the volume was set to 1.0 ml by tare, and 200 μl in triplicate was assayed for MI, and an additional 200-μl aliquot served as a blank. In order to destroy endogenous glucose, 20 μl of 0.1 N NaOH was added and the samples were heated for 20 min at 75°C. After cooling to room temperature, endogenous NADH was destroyed by addition of 20 μl 1.6 N HCL. After a 10 min incubation, 1,600 μl of 50 mM Na_2CO_3, pH 9.5, was added to each sample, followed by 200 μl of enzyme mixture. The enzyme mixture was freshly prepared and consisted of 0.24% bovine serum albumin, 0.5 U/ml myo-inositol dehydrogenase, and 6 mM NAD in 50 mM Na_2CO_3 (all from Sigma Chemical Co.). For the blank,

BSA and NAD were added, but not myo-inositol dehydrogenase. Myo-inositol standards were prepared in a final volume of 200 μl and ranged from 0 to 12 nmol. Fluoroescence was read at 1 h by which time the reaction had proceeded to completion. Excitation and emission wavelengths were 340 and 482 nm, respectively.

Gas chromatography-mass spectrometry (adapted and modified from references 38 and 39). A 1.0-ml sample of chilled plasma was treated with 1.0 ml of ice-cold 10% $HClO_4$ for 15 min. The mixture was vortexed every 5 min and the precipitated proteins pelleted by centrifugation. The supernatant was then treated with 5.0 N KOH until the pH was 7.0. After another centrifugation step, the supernatant was transferred to a 5.0-ml Wheaton vial and 12,500 ng of alpha-methyl-D-mannopyranoside was added as an internal standard (Aldrich Chemical Co., Milwaukee, WI). The mixture was dried down in a savant Speedvac concentrator (2 h at 45°C). The sides were then scraped with a small metal spatula, and 250 μl of trimethylsilating reagent was added (4.25 ml pyridine, 500 μl hexamethyldisilazane, and 250 μl trimethylchlorosilane; Aldrich Chemical Co.). After vortexing and sonication, the samples were allowed to incubate for 1 h at room temperature before a final centrifugation step. Standards containing 20, 50, and 100 ng/μl of myo-inositol and of alpha-methyl-D-mannopyranoside were run and yielded a linear relationship. A standard containing 50 ng/μl of myo-inositol and of alpha-methyl-D-mannopyranoside was processed concurrently with each batch of four to six samples. In this way a "relative response factor" of myo-inositol compared with the internal standard could be calculated, and subsequently used to determine the amount of myo-inositol in the sample. A Finnigan MAT (San Jose, CA) 4000 gas chromatography-mass spectrometry (GC/MS) system was used. The GC was a Finnigan model 9610 (Econocap SE-54 column, 30 meter × 0.25 mm inside diameter from Alltech Associates, Inc., Avondale, PA). Electron-impact ionization was employed. The sensitivity of the assay was 1 ng.

Aortic content of MI. 2-cm segments of freshly isolated aorta were thoroughly washed in ice-cold buffer free of myo-inositol. They were then homogenized in 1.0 ml 0.6 N $HClO_4$ and the supernatant neutralized with 5 N KOH. Myo-inositol content was measured by GC/MS as previously described for plasma.

Analytical procedure. Aortic protein was determined by the technique of Lowry et al. (40) using bovine serum albumin (Sigma Chemical Co.) as a standard.

Statistical analysis. Two factor univariate analysis of variance was used. Appropriate post-hoc tests, t tests and nonparametric tests were applied as needed. $P < 0.05$ was taken to be significant.

Results

Total water-soluble [³H]inositol phosphates. After various times of incubation with ³HMI, the basal accumulation of total [³H]inositol phosphates was significantly less in vasculature of late gravid rats, when compared with that of virgin controls (Fig. 1 and Table I). This finding held whether the data were normalized to milligrams wet weight or protein. Hormone-stimulated accumulation of [³H]inositol phosphates was also reduced in tissue from gravid animals (Table I). The ratio of hormone-stimulated to basal production of [³H]inositol phosphates (fold stimulation) by aortic vasculature was not significantly different between virgin and pregnant rats (Table II). When various dosages of arginine vasopressin were tested (10^{-9}–10^{-6} M), the ratio of stimulated to basal accumulation of total [³H]inositol phosphates in aortic vasculature was consistently similar between the two groups of animals ($n = 6$, virgin and pregnant rats for each dose, data not shown).

Individual water-soluble [³H]inositol phosphates. AVP (10^{-7} M) failed to stimulate production of [³H]inositol trisphosphate plus tetrakisphosphate at 45 s in aortic segments
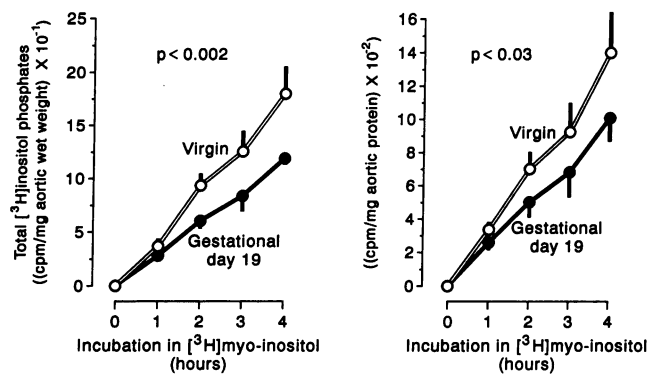
Figure 1. Time course of basal [³H]inositol phosphate production in aortic segments obtained from virgin and pregnant rats ($n = 7$ animals in each group). The tissues were loaded with [³H]myo-inositol for 1, 2, 3, or 4 h. For further details see Methods. Statistics were performed by two-factor ANOVA; the P values given indicate that basal production of total [³H]inositol phosphates was significantly less in tissues from gravid rats when compared to that of virgin controls. Standard error bars are depicted in this figure and in all subsequent figures.

from 3 of 10 virgin and 2 of 10 pregnant rats. Thus, these animals were omitted from the data portrayed in Fig. 2, *A* and *B*. Note that for the remaining rats, the basal production of [³H]inositol phosphates (time 0) was consistently less in aortic segments from pregnant animals, as we observed in the study of total water soluble [³H]inositol phosphates (Fig. 1 and Table I). After 45 s of stimulation with AVP, the absolute production of [³H]inositol phosphates was also less in vessels from gravid rats (Fig. 2 *A*, $P < 0.05$ vs. virgin for [³H]inositol monophosphate and [³H]inositol bisphosphate). This finding is again consistent with the study of total water-soluble [³H]inositol phosphates (Table I). However, the fold stimulation of [³H]inositol phosphates by AVP was also reduced for aortae from pregnant animals at the early time point of 45 s (Fig. 2 *B;* $P < 0.005$ vs. virgin for [³H]inositol monophosphate and for [³H]inositol trisphosphate plus tetrakisphosphate).

Fig. 3 *A* portrays the separation of the standards [³H]-InsP$_{1,3,4}$ and [³H]InsP$_{1,4,5}$ isomers as well as [³H]InsP$_{1,3,4,5}$ by HPLC. The 0.4-M ammonium formate fractions obtained from the Sep-Pak columns were applied to the HPLC. Figure 3 *B* depicts representative HPLC profiles of [³H]inositol phosphates synthesized by an aortic segment obtained from a virgin and pregnant rat. The vessels were stimulated with 10^{-7} M AVP for 30 s. Table III summarizes all of the data obtained by HPLC. At 30 and 60 s after addition of 10^{-7} M arginine vaso-

pressin, [³H]InsP$_{1,4,5}$ constituted $> 90\%$ of the total radioactivity in the 0.4 M ammonium formate fraction obtained from the Sep-Pak columns for tissues from both virgin and pregnant rats. Moreover, the absolute amount of [³H]InsP$_{1,4,5}$ produced by aortic segments from pregnant rats was consistently less than that synthesized by tissues from virgin animals, although in these experiments we did not measure basal levels.

Phosphoinositides, in vitro labeling. With a medium myo-inositol concentration of 0.47 μM, incorporation of [³H]myo-inositol into phosphoinositides of aortae obtained from rats at day 19 of gestation was significantly less than observed in tissues from virgin controls (Fig. 4, *A–D*). This finding was obtained whether results were normalized to milligrams wet weight or protein. The magnitude of the reduction in accumulation of total [³H]inositol phosphates from aortae of gravid rats discussed above (Fig. 1 and Table I) was comparable to the relative decline in [³H]myo-inositol incorporation into aortic phosphoinositides. Specifically, basal [³H]inositol phosphate production in vasculature of gravid rats was $73.4\pm6.4\%$ of that produced by aortae from virgin rats, and incorporation of [³H]myo-inositol into vascular phosphoinositides of gravid animals was similarly reduced after 3 h of incubation with the label (phosphatidylinositol-4,5-bisphosphate, $62.8\pm5.4\%$; phosphatidylinositol-4-phosphate, $57.7\pm8.3\%$; phosphatidylinositol, $70.3\pm8.5\%$).

When myo-inositol concentration in the medium was adjusted to 50–55 μM by adding unlabeled material, tissues from late pregnant animals still incorporated less [³H]myo-inositol than those of virgins (Table IV). Finally, the relatively deficient uptake of [³H]myo-inositol into phosphoinositides of aortae from gravid rats was observed whether the incubation medium consisted of EBSS or Ham's F12: Eagles Minimum Essential Medium, 1:1 (Table IV).

Fig. 5, *A* and *B* depict incorporation of [³H]glycerol into phosphatidylinositol and phosphatidylserine. In general, phosphatidylinositol incorporated considerably more label than phosphatidylserine. Norepinephrine further stimulated uptake into phosphatidylinositol, but not into phosphatidylserine. Overall, aortic segments from gravid rats incorporated $19\pm5\%$ less [³H]glycerol into phosphatidylinositol than vessels from virgin animals. On the other hand, uptake of the label into phosphatidylserine was comparable in vasculature from gravid and virgin rats.

Phosphoinositides, in vivo labeling. Dosages of [³H]myo-inositol were administered intravenously, such that plasma radioactivity was not significantly different in pregnant and virgin rats (Fig. 6 *A*). (Because endogenous plasma myo-inositol concentration was not significantly different between gravid

Table I. Total [³H]Inositol Phosphate Production in Aortic Segments Obtained from Virgin and Pregnant Rats*

	Basal	Norepinephrine	Basal	Arginine vasopressin	Basal	Angiotensin II
		$P < 0.01$		$P < 0.01$		$P = NS$
Virgin	1983±335	7877±760	2182±407	8402±1308	1735±319	2799±554
Pregnant	1513±81	5253±482	1031±112	4654±368	1247±67	1717±159

* Mean±SEM. Data are expressed as cpm/mg aortic protein. Six pregnant and six virgin rats were used. After two-factor ANOVA, Tukey tests were employed. When basal levels were combined, a significant difference was obtained by the Mann-Whitney U Test (pregnant: 1,264±156 cpm/mg aortic protein; virgin: 1,966±815 cpm/mg aortic protein [$P < 0.04$]).

| | Fold stimulation by vasoconstrictors | | |
	Norepinephrine	Arginine vasopressin	Angiotensin II
Virgin[‡]	3.63±0.27	5.23±0.64	1.50±0.11
Pregnant	3.33±0.28	4.70±0.33	1.45±0.08
Virgin[‖]	3.66±0.34	4.04±0.38	1.65±0.13
Pregnant	3.48±0.31	4.61±0.20	1.39±0.13

* Mean±SEM. The data from which these ratios were generated are displayed in Table I. No significant differences were observed between virgin and pregnant rats in the fold stimulation exerted by vasoconstrictors. [‡] The original data were expressed as cpm/mg aortic wet weight. [‖] The original data were expressed as cpm/mg aortic protein (see Table I).

and virgin rats, either (see below), the plasma specific activity was also comparable.) Uptake of [³H]myo-inositol was only detectable in lysophosphatidylinositol and phosphatidylinositol, the two most abundant phosphoinositides (Fig. 6 B). Compared with aortic segments from virgin rats, those from gravid animals incorporated 82±11% and 66±4% less label into lyso-phosphatidylinositol and phosphatidylinositol, respectively.

Myo-inositol uptake. The rate of uptake of [³H]myo-inositol by aortic vasculature from pregnant rats was 24% less than [³H]myo-inositol uptake by vessels from virgin animals, when medium myo-inositol was 50 μM (Fig. 7 A). Uptake of [³H]myo-inositol was completely inhibited in vessels from both

virgin and pregnant rats by removing K⁺ from the medium (Fig. 7 B) or by adding 1 mM ouabain (data not shown). Moreover, differences in uptake at 2 h were virtually abolished by incubating vessels from pregnant and virgin rats (n = 5 each) in 70 and 35 μM myo-inositol, respectively (data not shown).

Plasma concentration of myo-inositol. 1 ml of plasma was obtained from three virgin and three late pregnant rats. Using [³H]myo-inositol as a tracer, recoveries from the deproteinization procedure were comparable between plasma samples from pregnant and virgin animals and they averaged 62.6±2.7%. By enzymatic-fluorometric analysis, plasma myo-inositol concentrations were 162±10 and 173±9 μM in virgin and pregnant rats, respectively (n = 10 each). By GC/MS, values of 87±8 and 96±6 μM were observed (n = 19 each). Plasma concentrations of myo-inositol were not significantly different between virgin and gravid rats by either method of measurement.

Aortic content of myo-inositol. Using GC/MS, the myo-inositol content of aortic segments from virgin (n = 9) and gravid (n = 8) rats was 6.6±0.5 and 4.9±0.5 nmol/mg protein, respectively (P < 0.03). Compared with vessels from virgin animals, myo-inositol content was reduced by 22±6% in vessels from gravid rats.

Discussion

Vasodilation of the maternal circulation and attenuation of systemic pressor responsiveness are persistent features of normal human pregnancy. Chronically instrumented, conscious rats also manifest these physiological alterations during pregnancy (10–13). Therefore, the gravid rat may be a useful animal model in which to investigate mechanisms responsible for
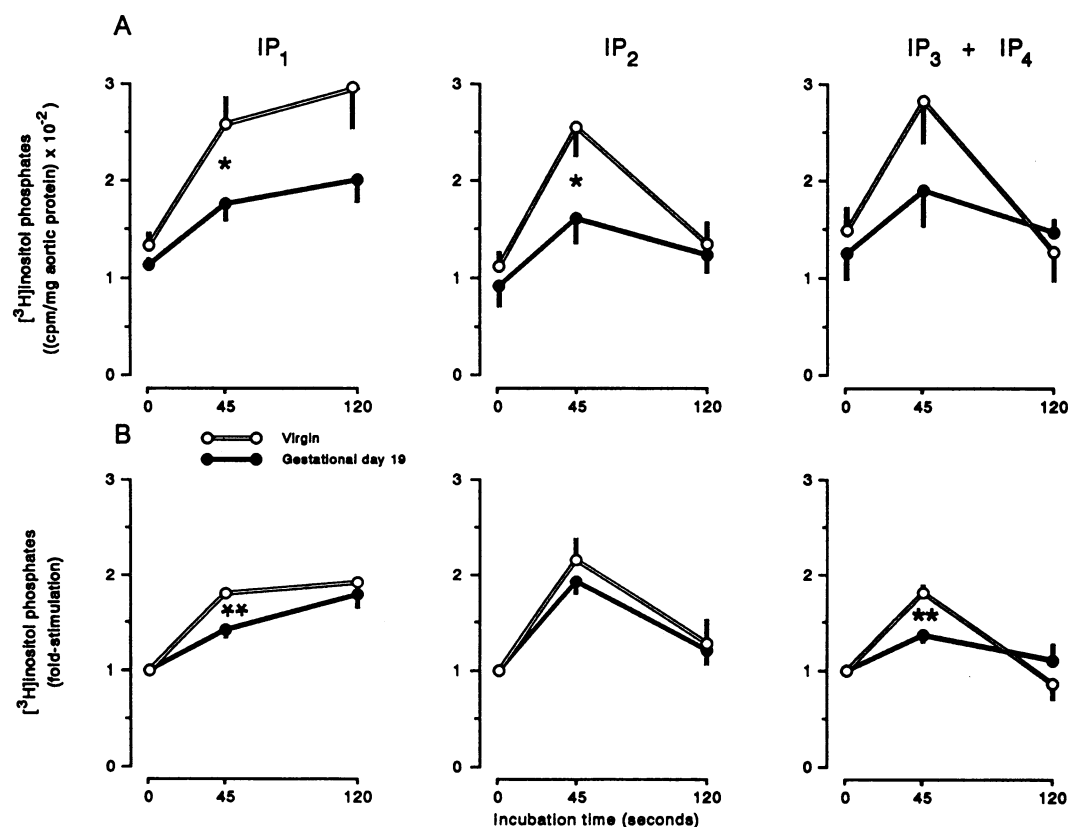

Figure 2. Time course of stimulation by 10^{-7}-M arginine vasopressin of individual water-soluble [³H]inositol phosphates in aortic segments from virgin (n = 7) and pregnant (n = 8) rats. See Methods for details. IP_1, inositol monophosphate; IP_2, inositol bisphosphate; and $IP_3 + IP_4$, inositol tris- plus tetrakis-phosphate. *P < 0.05, **P < 0.005 virgin vs. pregnant at the same time point by unpaired t tests.
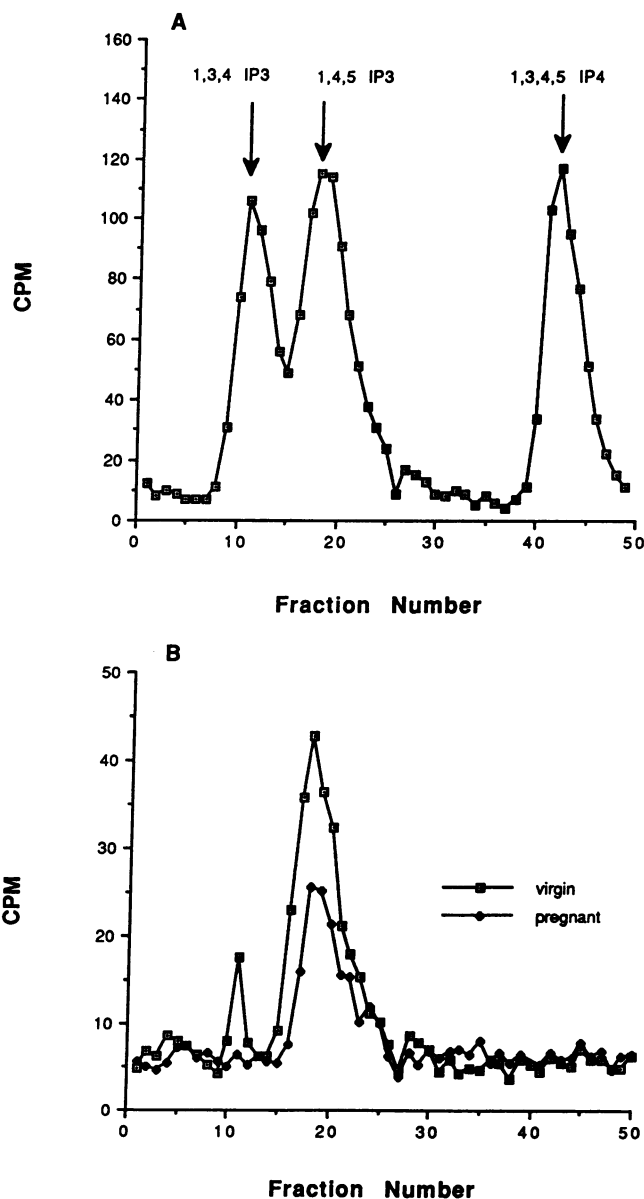
Figure 3. Representative HPLC analyses of [^{3}H]inositol phosphates synthesized by aortic segments in response to 10^{-7} M arginine vasopressin for 30 s. (*A*) A typical HPLC profile of a mixture of [^{3}H]-inositol phosphate standards applied to the column. (*B*) A representative experiment in which the IP$_3$ + IP$_4$ fractions from the Sep-Pak anion exchange columns were applied to the HPLC. See Methods for details.

maternal vasodilation. Most (14–20), but not all (41) investigators have demonstrated that the vasculature of gravid rats exhibits attenuated reactivity to vasoconstrictors in vitro or in situ. Conduit vessels including the aorta (14, 16, 19, 22), as well as resistance vessels (17, 20) develop attenuated reactivity during gestation. Further, the affinity and density of receptors responsible for mediating the response to vasoconstrictors during pregnancy have generally been reported not to be altered (23–26, 42). Vasodilatory prostaglandins may not underlie these changes in vascular response to vasoconstrictor administration (11, 22). These studies were designed to evaluate the hypothesis that alterations in postreceptor mechanisms that mediate vaso-

Table III. HPLC Profile of [^{3}H]Inositol Phosphate Synthesized by Freshly Isolated Aortic Segments in Response to 10^{-7} M Arginine Vasopressin

Condition of Rats	[^{3}H]Inositol phosphate			
	Time(s)	(1,3,4)IP$_3$	(1,4,5)IP$_3$	(1,3,4,5)IP$_4$
		cpm/mg aortic protein		
Pregnant	30	4	176	4
Virgin	30	15	247	8
Pregnant	30	0	114	0
Virgin	30	37	242	0
Pregnant	60	0	105	—
Virgin	60	0	116	0

* These data were gathered from three different virgin and pregnant rats. Aortic segments were labeled with 10 μCi/ml [^{3}H]myo-inositol for 3 h, incubated with 10^{-7} M AVP for 30 or 60s, extracted with perchloric acid, and the water-soluble inositol phosphates then separated by Sep-Pak chromatography. The inositol trisphosphate plus tetrakisphosphate fraction was further separated by HPLC. See Methods for details.

constriction, specifically of inositol lipid metabolism may account for vasodilation and reduced vascular reactivity that occur during pregnancy.

We first examined the production of total [^{3}H]inositol phosphates in the presence of 10 mM LiCl as an index of phosphoinositide hydrolysis. Because basal production was altered in vessels from gravid animals (see below), hormone-stimulated data were factored by basal values ("fold stimulation"). The fold stimulation by maximal doses of angiotensin II, arginine vasopressin, and norepinephrine was not significantly different between aortae from gravid and virgin rats (Table II). Moreover, the dose-response curves for arginine vasopressin obtained in vessels from both groups of animals were similar (see Results). These data suggest that activation of phospholipase C by vasoconstrictors is similar in aortae from pregnant and virgin rats. However, insofar as these experiments were conducted over a 15-min time period in the presence of LiCl, inositol monophosphate was probably the most abundant inositol phosphate measured. Comparatively smaller changes in InsP$_{1,4,5}$ produced at early time periods after addition of agonist were therefore overlooked. Because InsP$_{1,4,5}$ contributes to the initial rise in intracellular calcium critical for development of smooth muscle contraction, we next measured this product within seconds to minutes following agonist stimulation. 45 s after application of arginine vasopressin (10^{-7} M final concentration), the fold stimulation of [^{3}H]inositol tris- plus tetrakisphosphate was significantly less in vessels from gravid rats (P < 0.005 vs. virgin; Fig. 2 *B*). Further separation of [^{3}H]inositol tris- plus tetrakisphosphate by HPLC indicated that greater than 90% of the total radioactivity was in the form of InsP$_{1,4,5}$ (Fig. 3 and Table III). With the caveat that a reduction of radioactive InsP$_{1,4,5}$ does not necessarily imply a reduction in mass, the data suggest important biochemical differences are present in aortic tissue of virgin and pregnant rats.

Basal accumulation of [^{3}H]inositol phosphates was reduced in aortae of gravid rats (Fig. 1 and Table I). This finding could result from reduced labeling of phosphoinositides with [^{3}H]-myo-inositol (Fig. 4, *A–D*). Indeed, decreased labeling of the
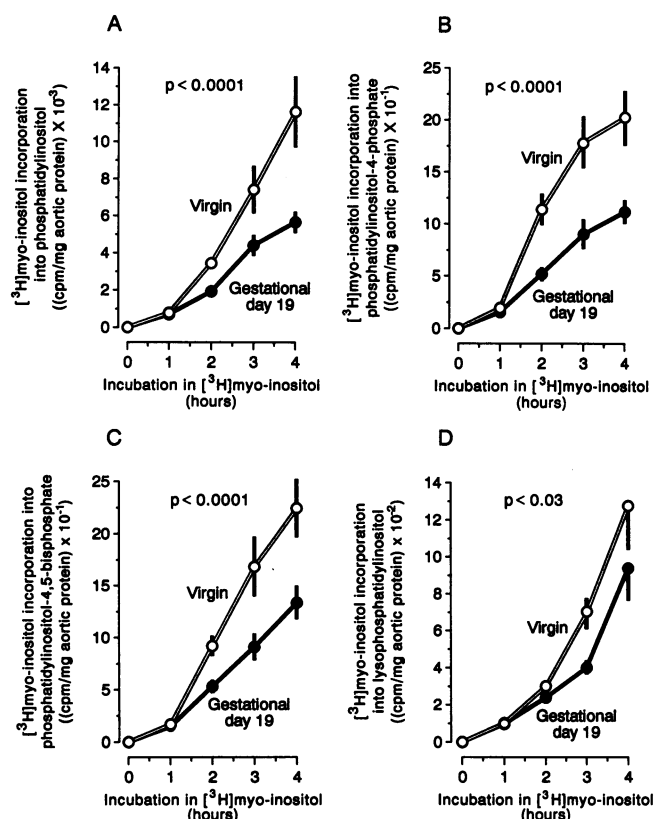
Figure 4. Time course of basal [³H]myo-inositol incorporation into phosphoinositides of aortic segments obtained from virgin and gravid rats (n = 9 animals in each group). The tissues were loaded for 1, 2, 3, or 4 h with [³H]myo-inositol (6 μCi/ml). Only labeled myo-inositol was added to the medium, so that the final concentration was 0.49 μM. See Methods for details. Statistics were performed by two-factor ANOVA; the P values given in the figures indicate that incorporation of [³H]myo-inositol was significantly less in tissues from gravid rats when compared with that of virgin controls.

phospholipids was uniformly observed, whether the medium in which tissues were incubated contained 0.49 μM myo-inositol or a more physiologic concentration of 50 μM, and whether

the medium used was a simple balanced salt solution or a cell-culture mixture (Table IV). When in vivo labeling experiments were performed in chronically instrumented conscious rats, significant reductions in incorporation of [³H]myo-inositol into phosphoinositides were again found in vessels from gravid animals (Fig. 6).

Possible explanations for diminished labeling of aortic phosphoinositides in gravid rats include: (a) attenuated cellular uptake of [³H]myo-inositol, and (b) decreased turnover of phosphoinositides. Decreased mass of phosphoinositides could also contribute to reduced [³H]myo-inositol incorporation. Before it can be incorporated into phosphoinositides, [³H]myo-inositol must first be transported into cells. Our results suggest that the mechanism of uptake in freshly isolated rat aorta is a Na⁺-cotransport process, insofar as [³H]myo-inositol uptake was completely inhibited in a K⁺-free medium or by 1 mM ouabain (see Results and Fig. 7 B). A Na⁺-cotransport mechanism for myo-inositol uptake has been reported for other tissues (reviewed in reference 43). We further observed that the rate of uptake of [³H]myo-inositol into vessels from gravid rats was 24% less than uptake by vessels from virgin animals (Fig. 7 A). Although reduced cellular uptake of [³H]myo-inositol undoubtedly contributed to deficient labeling of phosphoinositides, this explanation alone cannot account for the diminished labeling because the rate of incorporation of [³H]myo-inositol into phosphoinositides was reduced by a greater extent, approximately 50% (Fig. 4 and Table IV).

These results are compatible with the view that the turnover of phosphoinositides is attenuated in aortae of gravid rats. The fact that, in addition to reduced incorporation of [³H]myo-inositol, the uptake of [³H]glycerol into phosphatidylinositol (Fig. 5 A; but not into phosphatidylserine, Fig. 5 B) was also compromised, further supports the idea that phosphoinositide turnover is attenuated in vessels from gravid rats. Comparatively less incorporation of [³H]glycerol persisted even when uptake into phosphatidylinositol was stimulated by norepinephrine (Fig. 5 A).

In aortic vasculature from gravid rats, it is possible that reduced cellular uptake of myo-inositol limits the availability of intracellular myo-inositol for optimal synthesis of phosphatidylinositol. Consequently, basal as well as hormone-stimu-

Table IV. [³H]Myo-inositol Incorporation into Phosphoinositides of Aortic Tissue*

		Hours in [³H]myo-inositol				ANOVA	
		cpm/mg aortic wet weight					
		1.0	2.0	3.0	4.0	Virgin vs. pregnant	Time
	rats (n)						
Phosphatidylinositol	Virgin (7)	51.6±14.0	147.7±27.2	203.7±23.2	303.6±59.1	$P < 0.005$	$P < 0.001$
	Pregnant (7)	45.8±13.3	96.2±12.7	135.1±14.8	177.9±25.0		
Lysophosphatidylinositol	Virgin (7)	7.8±1.2	15.7±2.3	20.6±2.4	34.3±5.7	$P < 0.001$	$P < 0.001$
	Pregnant (7)	6.1±1.6	8.7±1.7	13.1±1.6	21.7±2.6		
Phosphatidylinositol-4-phosphate	Virgin (7)	1.7±0.4	5.3±0.8	8.6±1.5	9.8±1.9	$P < 0.01$	$P < 0.001$
	Pregnant (7)	1.3±0.2	4.3±0.3	5.9±0.9	6.0±0.9		
Phosphatidylinositol-4,5-bisphosphate	Virgin (7)	3.7±0.8	12.1±2.5	17.1±3.8	28.0±5.0	$P = 0.13$	$P < 0.001$
	Pregnant (7)	3.6±0.8	7.7±1.3	13.1±2.6	23.3± 4.7		

* Mean±SEM. Incubation media were Earle's Balanced Salt Solution (n = 4 rats) or Ham's F12: Eagles Minimal Essential Medium 1:1 (n = 3 rats) containing 50 and 55 μM myo-inositol, respectively. The results were similar and, therefore, combined. Pregnant rats were at day 20 of gestation.
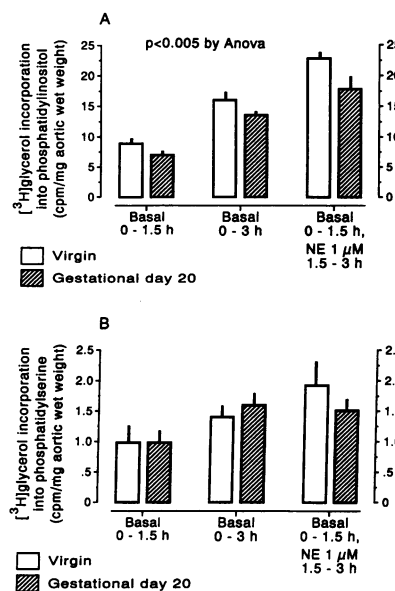
A

p<0.005 by Anova

[³H]glycerol incorporation into phosphatidylinositol (cpm/mg aortic wet weight)

□ Virgin
▨ Gestational day 20

Basal 0 - 1.5 h | Basal 0 - 3 h | Basal 0 - 1.5 h, NE 1 µM 1.5 - 3 h

B

[³H]glycerol incorporation into phosphatidylserine (cpm/mg aortic wet weight)

□ Virgin
▨ Gestational day 20

Basal 0 - 1.5 h | Basal 0 - 3 h | Basal 0 - 1.5 h, NE 1 µM 1.5 - 3 h

Figure 5. [³H]glycerol incorporation into phosphatidylinositol and phosphatidylserine of aortic segments obtained from virgin and gravid rats ($n = 9$ rats in each group). Three aortic segments were used from each animal. One of the segments was incubated with 6 µCi/ml [³H]glycerol for 1.5 h, a second segment for 3.0 h, and a third also for 3.0 h. The third segment, however, was exposed to 1 µM norepinephrine (*NE*) during the last 1.5 h of incubation. Medium concentrations of myo-inositol and glycerol were 50 and 100 µM, respectively. After loading of the aortic tissues, they were thoroughly washed, and phospholipids were extracted and separated by thin layer chromatography as described in Methods. Statistics were performed by two-factor ANOVA; the *P* value given in the figure indicates that overall, incorporation of [³H]glycerol into phosphatidylinositol was significantly less in aortae from gravid rats when compared with that in vessels from virgin controls.

lated turnover of phosphoinositides could be compromised. Indeed, measurement of aortic myo-inositol content by GC/MS demonstrated that vessels from pregnant rats contained $22\pm6\%$ less myo-inositol (see Results). Because the K_m for myo-inositol CDP-diacylglycerol: inositol phosphatidyltransferase may be quite high, ~ 4.6 mM (43), any reduction of intracellular myo-inositol could compromise synthesis of phosphatidylinositol and, thus, of polyphosphoinositides. Interestingly, the concept that reduced availability of myo-inositol could compromise phosphatidylinositol biosynthesis has been invoked to explain the change in phospholipid content of surfactant during lung maturation (44, 45), the neuropathy of diabetes (46), the salutory effect of Li⁺ in affective disorders (47, 48), as well as the accumulation of triacylglycerol in liver and intestinal lipodystrophy which occur with deficiency of dietary inositol (reviewed in reference 43).

The mechanism of reduced myo-inositol uptake by aortic vasculature of gravid rats is unknown. We considered the possibility that elevated plasma levels could downregulate the transporter. However, two independent methods of measurement failed to reveal significant differences in plasma myo-inositol concentrations between virgin and pregnant rats (see Results). In addition to alterations of the K_m or V_{max} of the Na/myo-inositol cotransporter, differences in Na/K ATPase activity could be responsible. Indeed, increased levels of circulating digitalis-like natriuretic factor have been reported in pregnancy (49). It would be of interest to determine if the defect in myo-inositol uptake that we observed is specific to vasculature, and whether other substances that undergo Na⁺-cotransport are also affected.

In summary, we have acquired evidence for attenuation of myo-inositol uptake, phosphoinositide turnover, and inositol
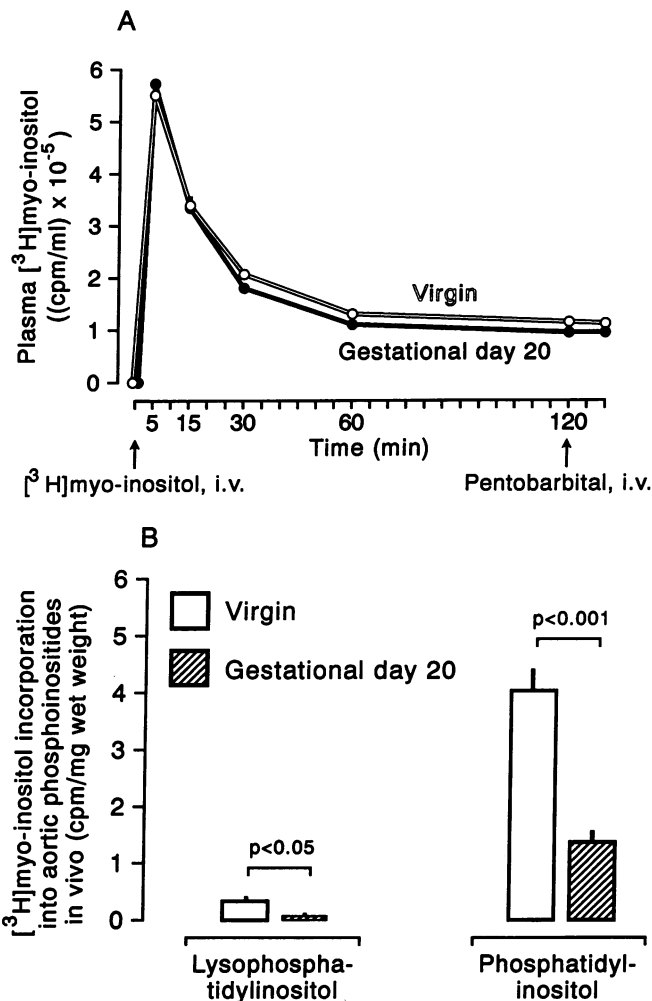
A

Plasma [³H]myo-inositol ((cpm/ml) × 10⁻⁵)

Virgin

Gestational day 20

Time (min)

[³H]myo-inositol, i.v. ↑   ↑ Pentobarbital, i.v.

B

[³H]myo-inositol incorporation into aortic phosphoinositides in vivo (cpm/mg wet weight)

□ Virgin
▨ Gestational day 20

p<0.05   p<0.001

Lysophosphatidylinositol   Phosphatidylinositol

Figure 6. [³H]myo-inositol incorporation into phosphoinositides of aortic segments in vivo. Four chronically instrumented virgin and pregnant rats were administered [³H]myo-inositol intravenously (50 µCi/275 g and 43 µCi/275 g body weight, respectively). Two-factor ANOVA revealed no significant difference in plasma radioactivity between virgin and gravid animals (*A*). 2 h after administration of [³H]myo-inositol, the animals were killed, the aortae removed, and analyzed for labeling of phosphoinositides (see Methods). (*B*) [³H]myo-inositol labeling of lysophosphatidylinositol and phosphatidylinositol. Significantly less incorporation of the label was observed in aortic phosphoinositides of pregnant animals (unpaired *t* tests).

phosphate production in aortic vasculature of rats during pregnancy. If these alterations also occur in resistance vessels, they could contribute to vasodilation and/or attenuated pressor responsiveness of pregnancy. A unifying theory is that reduced myo-inositol uptake is limiting intracellular availability of myo-inositol for optimal synthesis of phosphatidylinositol and, consequently, of polyphosphoinositides. On this basis, both basal and hormone-stimulated turnover of phosphoinositides and production of inositol phosphates could be compromised. On the other hand, multiple defects may be operative, e.g., alteration of G-protein function, activity of phospholipase C, or of other enzymes in the signal transduction pathway. Clearly, further investigation is needed to clarify this issue, as well as to identify the factor(s) responsible for initiating the
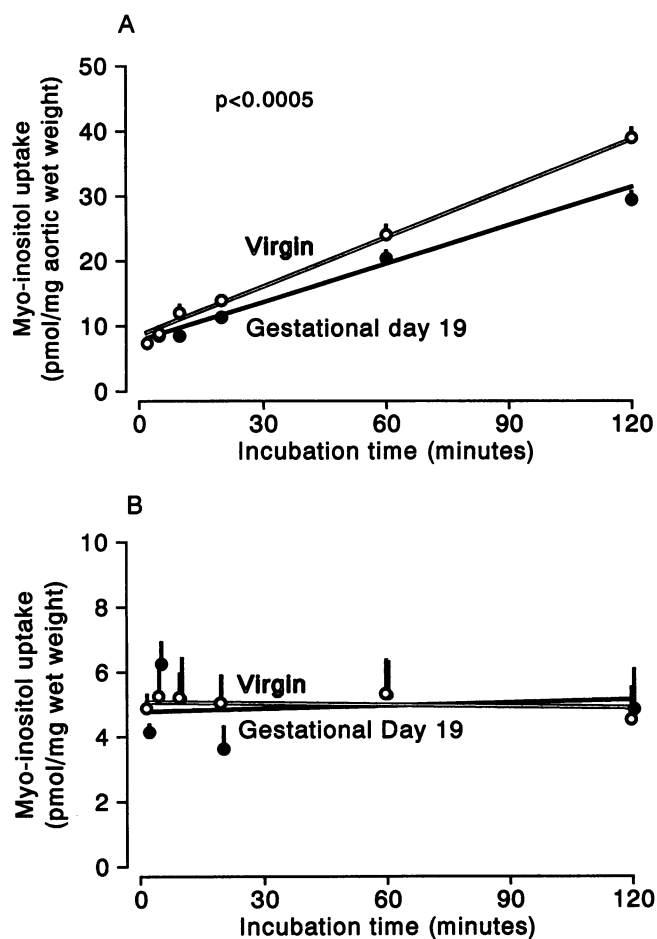
Figure 7. [^{3}H]myo-inositol uptake into aortic segments of virgin and pregnant rats ($n = 8$ animals each). Tissues were preincubated for 1 h in the presence (*A*) or absence (*B*) of K^+. The final concentration of myo-inositol was 50 μM. See Methods for details. Two-factor ANOVA was performed in *A;* the *P* value given in the figure indicates that overall, uptake of [^{3}H]myo-inositol was significantly less in aortic segments obtained from gravid rats, when compared with that of virgin controls. The slopes of the lines were 0.27±0.03 and 0.19±0.01 for virgin and pregnant animals, respectively ($P = 0.02$). In the absence of K^+ (*B*), little or no uptake was detected in aortae from either virgin or pregnant animals.

biochemical alterations observed in vasculature of pregnant rats.

Acknowledgments

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