Pregnancy and estradiol decrease GTPase activity in the guinea pig uterine artery

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Buhimschi, Irina A., Gentzon Hall, Loren P. Thompson, and Carl P. Weiner. Pregnancy and estradiol decrease GTPase activity in the guinea pig uterine artery. Am J Physiol Heart Circ Physiol 281: H2168–H2175, 2001.—The mechanisms by which pregnancy redistributes cardiac output in an organ-specific manner are poorly understood. We propose that it is consequential to estrogen-mediated alterations in G protein-mediated signal transduction. Aortas and uterine (UAs) and mesenteric arteries (MAs) were obtained from late-pregnant, nonpregnant, or ovariectomized guinea pigs chronically treated with 17β-estradiol. High-affinity GTPase activity was assayed enzymatically. The cGMP generated in response to the endothelium-dependent agonist ACh was measured in UAs incubated with or without cholera toxin (CTX, which inhibits Gαs). Pregnancy significantly decreased UA but not aorta or MA GTPase activity. 17β-Estradiol decreased UA GTPase activity compared with untreated ovariectomized animals. ACh increased cGMP in pregnant but not nonpregnant UAs. Pretreatment of nonpregnant UAs with CTX increased ACh-induced cGMP levels similar to pregnancy. Thus pregnancy and estradiol decrease the GTPase activity of a CTX-sensitive G protein in UAs, increasing receptor-dependent cGMP release. This alteration in receptor-mediated G protein coupling in UAs may contribute to the characteristic cardiovascular adaptation to pregnancy.

G proteins; nitric oxide; acetylcholine; estrogen

DURING NORMAL PREGNANCY, the maternal cardiovascular system undergoes profound changes crucial to accommodation of the demands of the growing conceptus. Both the maternal intravascular and stroke volumes increase by 45% by term (4, 15). Cardiac output increases similarly and is redistributed preferentially to vascular beds serving reproductively important organs. For instance, blood flow through the guinea pig uterine artery (UA) increases 3.500% by term, whereas flows through the mesenteric (MAs) and renal arteries (RAs) increase only 90% and 10%, respectively. Blood flow to the trunk actually decreases by term (21). The underlying cause for this redistribution of cardiac output among reproductively important and unimportant vessels is largely unknown. Studies of isolated blood vessels employing pharmacological manipulations document regional changes in responsiveness to vasoactive agents typically consistent with the changes in blood flow through those vessels (32). For example, pregnancy decreases tone by both decreasing arterial sensitivity to vasoconstrictors (e.g., angiotensin II, norepinephrine, and epinephrine) (1, 2, 7, 22, 29) and/or by increasing the release of endothelium-derived vasodilators such as nitric oxide (NO) (30), its secondary messenger, cGMP, or PGI2.

Contractile responses of isolated vessels to norepinephrine (27, 33), thromboxane, and angiotensin II are also reduced during pregnancy (31) and by estradiol treatment (16). Such changes are again most pronounced in the UA (29). Stimulation of muscarinic receptors by ACh (6) and bradykinin receptors by bradykinin induces the release of endothelial-derived NO (10) by activating the endothelial isozyme of NO synthase (eNOS). During pregnancy, receptor-mediated release of NO by UA is increased both in guinea pigs (30) and humans (18). Furthermore, the sensitivity of UA to ACh is also enhanced, but to a lesser extent than for UA, whereas the RA remains unaffected (12), mirroring the changes in blood flow through these arteries during pregnancy.

Across species, there is a net increase in the endothelial production of NO in UA, cGMP (11, 14, 19, 28, 33), and PGI2 (8, 13) during pregnancy paralleled by increased expression of the synthesizing enzymes (11, 14, 18). Although heterogeneity among species and autacoids exists, recent experimental evidence from our laboratory and others contradicts the popular premise (32) that pregnancy alters the responsiveness to constrictors simply by increasing the release of endothelium-derived dilators (NO and/or PGI2) (27, 28). First, this laboratory has demonstrated that, although pregnancy and estradiol increase calcium-dependent NOS activity and eNOS and neuronal-derived NOS mRNA transcription, the increase is global (throughout the body), which certainly does not mirror the change in blood flow (28). In fact, NOS activity is increased during pregnancy and after estradiol administration to an extent similar to that to the UA in many organs where blood flow does not rise during pregnancy. The effect of pregnancy and estradiol on eNOS

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expression contrasts with the vessel and organ-specific, receptor-stimulated release of NO, suggesting that an increase in NO production is not a sufficient explanation. Second, it is only receptor-mediated release of endothelium-derived NO that is enhanced by pregnancy, because UA response to both native NO and the calcium ionophore A-23187 (that stimulates eNOS) by a receptor-independent mechanism are unaltered by pregnancy (27, 33). Third, pregnancy induces refractoriness to several vasoconstrictors (serotonin and thromboxane) in some species by a mechanism that cannot be explained by increased NO or PGI2 (12, 17, 31). Fourth, although pregnancy seems to enhance the net vasodilatory response overall, there are well-characterized exceptions where the contractile responses of some vessels are enhanced by pregnancy (32). Together, these findings suggest that, although both basal and stimulated release of NO and/or PGI2 are increased in many vascular beds during pregnancy, they lack specificity and cannot provide the unifying mechanism for cardiovascular adaptations of pregnancy. We have further observed a feature of vasoactive responses altered by pregnancy (reported either by us or by other investigators) is that they involve autacoid binding to receptors coupled to their effector apparatus by G proteins. Consequently, we proposed that pregnancy alters the cross talk between vasoactive mediators (both dilators and constrictors) and multiple effectors by specifically targeting G proteins (32).

Recently, we demonstrated that the pretreatment of UA from nonpregnant guinea pigs with cholera toxin (CTX) duplicates the ACh relaxation profile of UA from pregnant guinea pigs with cholera toxin (CTX) (5 mo) were obtained from a commercial breeder (Harlan (64x148) to directly measure guinea pig vascular GTPase activity. Consequently, we proposed that pregnancy alters the cross talk between vasoactive mediators (both dilators and constrictors) and multiple effectors by specifically targeting G proteins (32).

Measurement of GTPase activity. GTPase activity was assayed as described first by Cassel and Selinger, modified by Sokolovsky (24), and further optimized as described below. In one experiment, UA from four nonpregnant animals were dissected under the microscope from the surrounding tissue and cut into rings of a relative consistent size, and an equal number of rings of arteries from each animal were placed in tubes containing 1 ml of Krebs buffer with 2 μg/ml CTX. The tubes were then placed for 1 h in a 37°C water bath, oxygenated, and buffered by slowly bubbling a 95% O2-5% CO2 gaseous mixture into the Krebs solution containing the tissues. After incubation, the rings were removed from the medium and flash-frozen in liquid nitrogen.

Crude membrane fraction preparation. Frozen tissues (whole aorta and both UAs and the MA from each animal or incubated rings) were pulverized in liquid nitrogen and homogenized in 2 ml of 10 mM triethanolamine-HCl and 140 mM NaCl, pH = 7.4, and centrifuged at 3,500 g for 20 min at 4°C. The pellets were suspended in the same buffer and centrifuged at 4,300 g for 20 min at 4°C. The final pellet was then resuspended in a buffer containing 0.25 M sucrose, Tris-HCl, pH = 7.5, 5 mM MgCl2, 1 mM ATP, and protease inhibitors (100 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotenin, and 1 mM dithiothreitol) and filtered through three layers of cheesecloth. The resulting supernatant was centrifuged at 15,000 g for 20 min at 4°C. The membrane pellet was washed three times and uniformly resuspended at 0.5 mg protein/ml by vigorous shaking in a Savant Fastprep Bio101 homogenizer (Savant Instruments; Farmingdale, NY). The protein content was measured with a BCA kit (Pierce; Rockford, IL) against albumin standards.

Measurement of GTPase activity. Hydrolysis of [γ-32P]GTP (0.1 μCi/tube) was carried out at 37°C and pH = 7.3 in 120 μl reaction mixtures containing 50 mM triethanolamine-HCl, 1 mM EDTA, 0.5 mM ATP, an ATP regeneration system (5 mM creatine phosphate, 0.4 mg/ml creatine kinase), 100 μg/ml BSA, 5 mM MgCl2, and either 1 μM (total activity reaction buffer) or 50 μM GTP (low affinity reaction buffer). Duplicate reactions were initiated by the addition of the membranes (10 μg protein) to prewarmed reaction mixtures, allowed to proceed for 12 min at 37°C and terminated by the addition of 0.7 ml ice-cold sodium phosphate buffer (10 mM, pH = 2.0)
containing 5% (wt/vol) activated charcoal. After incubation for 10 min at 0°C, the reaction mixture was cleared by low-speed centrifugation and radioactivity ([32P]Pi) was measured in 0.5 ml of the supernatant. Reactions without protein (blanks) and reactions without protein and charcoal (total counts) were run in parallel in the two incubation buffers. The counts obtained from the blank reactions with the respective buffer were subtracted from the average counts of the duplicate sample reactions. High specific GTPase activity was calculated by subtracting the activity of the low affinity reaction (activity in the presence of 50 μM GTP) from the total reaction activity (activity in the presence of 0.1 μM GTP), and then normalized for total count values to correct for interassay variability. Because we observed that radioisotope decay was the central determinant factor of interassay variability, all samples were run simultaneously within one assay to minimize error for comparisons among groups. The calculated intra-assay and interassay variations were 6.9 and 36%, respectively. The GTPase activity was expressed as picomoles [32P]Pi per milligram protein per minute. In preliminary experiments, we determined that high specific GTPase activity was achieved in triplicate using a commercially available [3H]cGMP assay (Amersham; Arlington Heights, IL). The protein pellet was resuspended in 0.5 ml of 0.1 mM NaOH and the total protein content was measured with the BCA kit. Finally, the cGMP content was normalized for the incubation volume and protein content, and then expressed as either pmols/mg protein, or as the percent from basal level (for CTX incubation experiments).

**Statistical analysis.** All data sets were tested for a normal distribution (Kolmogorov-Smirnov) before parametric tests were performed. The results are presented as means ± SE. Statistical comparisons between two groups were performed using Student’s t-test and among multiple groups, using one-way or two-way ANOVA followed by post hoc testing with Student-Newman-Keuls tests. A two-tailed P < 0.05 was considered indicative of statistical significance.

**RESULTS**

**Vascular GTPase activity.** Pregnancy significantly decreased GTPase activity in UA (nonpregnant 24.7 ± 5.5 pmol [32P]P-i mg⁻¹ min⁻¹ vs. pregnant 11.8 ± 2.0 pmol [32P]P-i mg⁻¹ min⁻¹, P = 0.03, n = 9–11 per group) but not in the aorta (nonpregnant 17.7 ± 3.1 pmol [32P]P-i mg⁻¹ min⁻¹ vs. pregnant 15.9 ± 2.0 pmol [32P]P-i mg⁻¹ min⁻¹, P > 0.05, n = 7–8) (Fig. 1). Similar to the aorta, MA GTPase activity was unaltered by pregnancy. Chronic 17β-estradiol replacement at doses replicating pregnancy (25) significantly reduced GTPase activities in UA from ovariectomized guinea pigs at each estradiol dose studied (Fig. 2). Untreated ovariectomized animals had the highest GTPase activities (104.2 ± 14.0 pmol [32P]P-i mg⁻¹ min⁻¹, n = 5), approximately four times the nonpregnant CRL. Conversely, the 0.1- and 0.25-mg 17β-estradiol pellets induced UA GTPase activities similar to pregnancy. Although there were no statistically significant differences achieved among the estradiol doses with the sample size, animals treated with the 0.5-mg 17β-estradiol pellets had significantly higher UA GTPase activity (27.0 ± 7.80 pmol [32P]P-i mg⁻¹ min⁻¹, n = 5) compared with pregnant animals (11.8 ± 2.0 pmol [32P]P-i mg⁻¹ min⁻¹, P = 0.02).

Figure 3 illustrates the changes in GTPase activity after receptor activation with the endothelium-dependent agonists ACh and bradykinin. There is a statistically significant difference in GTPase activity for both agonists by two-way ANOVA for pregnancy status, although the pregnancy effect is in the opposite direction for the aorta and UA (UA/ACh F = 38.4, P < 0.001; UA/bradykinin F = 14.8, P = 0.001; aorta/ACh F = 7.8, P = 0.009; and aorta/bradykinin F = 6.3, P = 0.16). ACh (all doses studied) and bradykinin (10⁻⁷ M) significantly increased GTPase activity solely in UAs from nonpregnant animals. During pregnancy, however, GTPase activity decreased in UAs, increased in aortas, and was unchanged in MAs.

**Vascular cGMP production.** cGMP levels were 10-fold higher in UAs compared with either MAs or aortas independent of pregnancy status (Fig. 4). ACh significantly increased cGMP only in UAs from preganant animals (nonpregnant 78.6 ± 15.0% from CRL vs. pregnant 131.9 ± 7.8% from CRL, P = 0.03, n = 8 in
each group) (Fig. 5). However, CTX abolished the difference in cGMP generation between the UAs from pregnant and nonpregnant animals. Specifically, UAs from nonpregnant animals incubated with CTX produced higher cGMP levels and no longer differed from that produced by UAs from pregnant animals (nonpregnant 160.8 ± 17.1% from CRL vs. pregnant 164.5 ± 15.5% from CRL, \( P < 0.05, n = 4 \) in each group) (Fig. 5). The bioavailability of CTX to vascular GTPase was confirmed by the measurement of GTPase activity in the rings exposed to CTX. In vitro exposure of nonpregnant uterine arteries to CTX significantly decreased GTPase activity to 59.8 ± 11.7% of basal (\( P = 0.02, n = 4 \)).

**DISCUSSION**

G proteins mediate the flow of information from membrane-bound receptors (more than 1,000 receptors are known to be G protein coupled) to a variety of intracellular effectors including enzymes (e.g., guanylate and adenyl cyclases, cGMP phosphodiesterases, and phospholipase A2 and C) and ion channels (e.g., Ca\(^{2+}\)-activated potassium channels). Each G protein is a heterotrimer composed of an \( \alpha \)-subunit bound noncovalently to a tightly coupled dimer consisting of a \( \beta \)- and a \( \gamma \)-subunit (3). The \( \alpha \)-subunit has intrinsic GTPase activity. G proteins recycle between an active GTP-ligated state and an inactive GDP-ligated state when the flow of information to the effector(s) is interrupted. There are four major families of G proteins (Gs, Gi, Gq, and G12) based on the identity of various \( \alpha \) subunits; each regulates distinctive, downstream signaling pathways. Multiple interactions between the activated G proteins and their effectors provide considerable opportunity for amplification of the primary stimulus. Thus a change in the G protein coupling mechanism related to either pregnancy or sex hormones could have a dramatic effect on the response of a large array of receptors without a change in receptor number or binding. Their central regulatory position renders G protein coupling mechanisms ideal regulatory sites for the concerted alteration of responses to multiple agonists. The role of G proteins can be investigated using specific agents that interfere with one or more steps in the coupling-uncoupling cycle. For example, the function of Gs proteins is irreversibly altered by CTX, whose binding prevents access of the high-affinity GTPases to the GTP-ligated activated complex, thus prolonging the half-life of the activated complex.

![Graph A](image1.png)

**Fig. 1.** Basal high-affinity GTPase activity in uterine arteries (UAs) (A), aorta (B), and mesenteric arteries (MAs) (C) from pregnant (P) and nonpregnant (NP) guinea pigs. Data are presented as means ± SE. The number of animals in each group are as follows: \( n = 11 \) (NP UAs), \( n = 9 \) (P UAs), \( n = 7 \) (NP aortas), \( n = 8 \) (P aortas), and \( n = 5 \) (P and NP MAs). *P UA is statistically different at a value of \( P < 0.05 \) from NP UAs (Student’s t-test).

![Graph B](image2.png)

**Fig. 2.** Basal high-affinity GTPase activity in UAs from ovariectomized (OVX, \( n = 4 \)) and chronically estrogen-replaced guinea pigs implanted with cholesterol-based pellets releasing 0.1 (\( n = 4 \)), 0.25 (\( n = 3 \)), or 0.5 mg (\( n = 4 \)) 17β-estradiol over a 21-day period. Data are presented as means ± SE. *OVX is statistically different at a value of \( P < 0.05 \) (one-way ANOVA followed by post hoc Student-Newman-Keuls tests).
The results of this investigation indicate that first, pregnancy and chronic treatment with 17β-estradiol significantly decrease high-affinity GTPase activity in UAs but not aortas or MAs. Second, the basal rate of cGMP production by UAs is higher than aortas or MAs independent of pregnancy. However, pregnancy significantly augments cGMP generation in response to the muscarinic receptor agonist ACh, which in the guinea pig UA triggers the release of only endothelium-derived NO. Third, the in vitro treatment of UAs from
The basal cGMP production in UAs, although significantly higher compared with reproductively unimportant arteries (such as MAs or aortas), does not differ by pregnancy status. However, the cGMP production of UAs in response to muscarinic receptor stimulation is significantly increased during pregnancy. Furthermore, exposure of UAs from nonpregnant animals to CTX increases endothelium-dependent cGMP production to a degree similar to that found during pregnancy. The cGMP levels measured in UAs during the present study are also consistent with our prior reports (18, 30, 33) of increased NOS activity in UAs from pregnant guinea pigs and increased sensitivity of UAs to ACh during pregnancy while confirming the effectiveness of our toxin treatments in vitro (Fig. 4). The latter is important as these toxins reportedly have poor membrane penetrability in whole tissues and may require the addition of cofactors, especially when subcellular fractions are used. Our results indicate this possibility was not a problem. Furthermore, in the cascade of events triggered by ACh and ending in relaxation, our results demonstrate that there is indeed a CTX-sensitive G protein coupled to muscarinic receptors whose activation in guinea pig UA triggers NO/cGMP synthesis. Most likely, the regulated effector is eNOS and not guanylate cyclase, which is localized in the underlying smooth muscle a distance from endothelial receptors.

Presently, we do not know the nature of the subunit protein whose GTPase activity is downregulated or the mechanisms by which this phenomenon occurs. Its CTX sensitivity suggests a Gs subunit is involved. It is of note that there is a Gs subunit within the endothelial caveolae in close proximity to eNOS (23). It is possible that pregnancy and estradiol lower the expression of the Gs subunit and, as a result, the measured GTPase activity intrinsic to the a-subunit. However, conceptually, this mechanism seems unlikely, because G proteins are structurally needed to relay the flow of information even if their turnover is delayed. Alternatively, we suggest pregnancy may alter the regulatory site where the activated complex interacts with the GTPase-activating proteins (GAPs) or regulators of G protein signaling (RGS), members of a recently discovered family of proteins that are GAPs for heterotrimeric G proteins. RGS proteins enhance the time resolution of G protein signaling cascades by accelerating the hydrolysis of GTP Gα subunits. Our present findings are consistent with the specific downregulation in UA of an RGS protein by pregnancy and estradiol. In nonpregnant animals with CTX in a protocol that effectively inhibits GTPase activity significantly increases cGMP production after ACh stimulation. As a result of CTX pretreatment, cGMP production in UAs from nonpregnant animals is similar to UAs from pregnant animals. In contrast, the ACh-induced cGMP of UAs from pregnant animals is unaltered by CTX, suggesting that the GTPase activity is already sufficiently inhibited by pregnancy. These findings closely parallel our studies of isolated UAs (26). These findings demonstrate that pregnancy and estradiol alter G protein-mediated signal transduction in UAs by selectively targeting GTPase activity. A decrease in GTPase activity prolongs the half-life of the activated G protein-GTP complex, facilitating downstream signal transduction, releasing endothelium-dependent vasodilators.

UAs from nonpregnant animals had higher GTPase activity compared with UAs from pregnant animals, and this activity was significantly reduced by either CTX or agonist-mediated receptor activation. Furthermore, the effects of chronic estradiol replacement on GTPase activity were analogous to the effects documented for pregnancy. GTPase facilitates deactivation of the downstream signaling pathway by rendering agonist binding to the receptor inefficient. Thus a decrease in GTPase activity (by any mechanism) would prolong both the time the Gα subunit stays bound to its effector and the time the downstream pathway is efficiently activated. Consequently, the downstream result for any given G protein-coupled agonist will be dramatically different even with the same agonist concentration and receptor binding. These findings support our prior biomechanical studies (26) of isolated arteries and provide direct evidence for our hypothesis that there is a reduction in GTPase activity in UAs from pregnant and estradiol treated animals. The results are also consistent with D’Angelo and Osol (5), who reported that G proteins of arcuate arteries from nonpregnant rats are more susceptible to deactivation.

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support of this possibility, it was reported that RGS proteins modify the signaling of the M₃ muscarinic cholinergic receptor within caveolae (9). Whether such an interaction exists in the endothelium is unknown. In other instances, the RGS domains signal between different Go-coupled signaling pathways and, in some cases, serve to integrate small GTPases with the G protein signaling pathways. There is additional GTPase activity within endothelial caveolae not associated with heterotrimeric G proteins, but rather with small GTPases (such as Ras). This GTPase activity is usually low, but can be markedly increased by interacting with specific GAPs (20). This nonheterotrimeric GTPase activity could well be a component of the basal GTPase activity measured in this study.

The present investigation was limited to ACh/bradykinin-G protein interaction. However, we believe there is abundant information that if pregnancy and estradiol alter the half-life of the G protein-coupled activated complex, it could explain most of the reported cardiovascular adaptations induced by pregnancy.

Previous studies (28, 32) by our group have demonstrated that pregnancy and estradiol stimulate a global increase in guinea pig calcium-dependent NOS activity. We have also demonstrated that pregnancy increases the stimulated release of endothelium-derived NO from the UA of the guinea pig by receptor-mediated stimuli such as ACh and bradykinin (12, 30, 31). In contrast, pregnancy has no effect on UA relaxation to either native NO (27, 33), the calcium ionophore A-23187 (27), or 8-bromo-cGMP, suggesting the changes in vascular reactivity induced by pregnancy occur at the level of endothelial NO release. Subsequently, we found indirect evidence that the mechanism by which pregnancy-enhanced UA NO release was associated with altered G protein-mediated signal transduction (26). In these studies, we also demonstrated that the sensitivity of UA to sodium fluoride, which acts to cause relaxation by nonspecifically activating G proteins independent of receptors is significantly increased during pregnancy. Furthermore, we reaffirmed prior reports (12, 30, 31) that the sensitivity and maximal dilatory response of UA from pregnant guinea pigs to ACh is dramatically increased compared with UA from nonpregnant animals. When the UAs from nonpregnant animals were pretreated with CTX, relaxation of UAs from nonpregnant animals to ACh was dramatically increased and no longer different from UAs obtained from pregnant animals. It may be noteworthy that the effect of bradykinin on GTPase activity appears biphasic, which parallels its effect on vascular reactivity. We observed that GTPase activity is increased in the pregnant aorta after 10⁻⁷ M bradykinin, suggesting a higher turnover of the G protein-effector complex and less downstream signaling. This finding is reversed in the UA, where GTPase activity is significantly lower after 10⁻⁷ M bradykinin, suggesting increased responsiveness to this agonist. The effect may even be larger compared with the nonpregnant state, because there is an intrinsic increase in GTPase activity after both ACh and bradykinin in the nonpregnant animal compared at least to the aorta and MA. Thus the decrease in GTPase brought about by pregnancy is relatively larger in UA compared with the MA and aorta.

It is also possible that the cardiovascular adaptations characteristic of pregnancy also involve an alteration in either receptor number or binding affinity. We believe this is less likely, because CTX essentially eliminates the differences in cGMP production and relaxation responses (26) to ACh in UAs from pregnant and nonpregnant guinea pigs.

In conclusion, pregnancy and estradiol induce changes in a G protein-mediated signal transduction pathway within the endothelium of selected blood vessels (such as UAs) consistent with a decrease in GTPase, which in turn is responsible for an increased rate of cGMP production and vasorelaxation in response to ACh. Although the means for this alteration have yet to be determined, it could provide a unifying mechanism to account for the cardiovascular adaptations of pregnancy. If true, its elucidation would open new areas of investigation and therapeutic intervention for the treatment of poor adaptive responses during human pregnancy (e.g., preeclampsia and intrauterine growth restriction), which could be explained by differences in the G protein apparatus among women.

REFERENCES


