Pregnancy alters nitric oxide synthase and natriuretic peptide systems in the rat left ventricle

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Abstract

Cyclic guanosine monophosphate (cGMP), which is implicated in cardiac cell growth and function, is synthesized by cytoplasmic soluble guanylyl cyclase (GC) stimulated via nitric oxide (NO) and by particulate membrane-bound GC activated via natriuretic peptides. We investigated possible cGMP elevation in the left ventricle (LV) of rats developing physiologic LV hypertrophy during gestation. Furthermore, expression of estrogen receptors (ER) and oxytocin receptors (OTR) was evaluated because their activation stimulates NO and atrial natriuretic peptide (ANP) release from the heart. Compared with nonpregnant controls, Sprague-Dawley rats on day 7 of gestation had similar heart weights, but, on days 14 and 21, ventricular mass increased by 12% and 28% respectively ($P<0{\cdot}05$). LV cGMP concentration was elevated at day 14 of gestation ($3{\cdot}25 \pm 0{\cdot}12$ vs $4{\cdot}65 \pm 0{\cdot}17$ pmol/g wet weight, $P<0{\cdot}01$) but decreased at day 21 ($2{\cdot}45 \pm 0{\cdot}09$ pmol/g, $P<0{\cdot}05$) to increase again on postpartum day 1 ($6{\cdot}01 \pm 0{\cdot}15$ pmol/g) and day 4 ($9{\cdot}21 \pm 1{\cdot}79$ pmol/g). Changes in endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), OTR and ERα, but not ERβ, proteins paralleled the pregnancy-related cGMP changes in the LV. In contrast, ANP mRNA of the LV remained at control level throughout gestation but increased postpartum, whereas brain natriuretic peptide (BNP) expression declined at term and increased postpartum. The particulate GC natriuretic peptide receptors (GC-A and GC-B) transcripts were already lower at day 14 of gestation. Natriuretic peptide clearance receptor (NPR-C) transcript was not altered on days 7 and 14, but increased at term. We conclude that cGMP concentration in the rat LV is influenced by both NOS and natriuretic peptide systems and may be involved in the changes of LV contractility and hypertrophy that occur during rat gestation.

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Introduction

In the rat, maternal cardiovascular adaptation to pregnancy is initiated 2 days after implantation and is manifested by expanded stroke volume as well as increased heart contractility (Slangen *et al.* 1997). In effect, pregnancy triggers physiologic left ventricular (LV) hypertrophy and atrophy secondary to a transient, self-limited hemodynamic load, making the heart mechanically more efficient. The importance of these alterations becomes evident in preeclampsia when cardiac hypertrophy is exaggerated, and its resolution remains incomplete after parturition (Simmons *et al.* 2002).

In response to elevated female steroid hormones during gestation, cardiac growth and contractility are modulated profoundly in parallel with changes in various hormones, electrolyte balance, blood volume and blood pressure (Granger 2002). Estrogens regulate cardiac hypertrophy by direct effects on the heart and by triggering the release of cardioprotective factors. Estrogen stimulates nitric oxide synthase (NOS) in various organs, including cardiac tissue, in association with an increase of cyclic guanosine monophosphate (cGMP) (Weiner *et al.* 1994), the second messenger of nitric oxide (NO). Three different isoforms of NOS catalyze the oxidation of L-arginine to citrulline and NO: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) NOS. Myocardial eNOS appears to be important under physiologic conditions in pregnancy, where it is involved in cardiac hypertrophy (Trochu *et al.* 2000). eNOS knockout mice develop the hypertrophic phenotype by 5 months of age, manifested as increased wall thickness (Barouch *et al.* 2002). Interestingly, cGMP levels in the LV are unaffected by eNOS gene knockout because of compensation of its synthesis by upregulated atrial natriuretic peptide (ANP) (Gyurko *et al.* 2000), indicating an interaction between NOS and natriuretic peptide systems in the LV.

ANP and brain natriuretic peptide (BNP) are mainly produced in cardiac atria and ventricles respectively, and both are released into the circulation and influence

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blood volume and pressure. Cardiac ANP expression is influenced by elevated estradiol during pregnancy and postpartum (Jankowski *et al.* 2001, Mukaddam-Daher *et al.* 2002). Brown *et al.* (1993) have shown that the transcriptional downregulation of heart natriuretic peptide receptors is associated with the development of hypertrophic cardiac pathology in the rat. Two natriuretic peptide receptors, guanylyl cyclase (GC)-A and GC-B (also known as NPR-A and NPR-B), are signal-transducing, single-pass transmembrane glycoproteins exhibiting ligand-dependent intrinsic GC activity (Tremblay *et al.* 2002, Kuhn 2003). ANP and BNP have been shown to bind preferentially to GC-A, while C-type NP (CNP) displays a greater affinity for GC-B (Koller *et al.* 1991, Suga *et al.* 1992). A third receptor, NPR-C, lacks the GC domain, and modulates natriuretic peptide concentration through local metabolism (Nakao *et al.* 1993). The GC-A system has intrinsic growth inhibitory properties in cardiac fibroblasts (Cao & Gardner 1995), and ANP and BNP inhibit cardiac ventricular myocyte hypertrophy to a greater extent than NO (Horio *et al.* 2000), independently of blood pressure (Kishimoto *et al.* 2001). It is noteworthy that inactivation of GC-A, resulting in hypertension, cardiac hypertrophy and increased mortality, evokes gender disparity, suggesting a protective action of estrogen (Vuolteenaho & Ruskoaho 2003). Although the natriuretic peptide system is recognized as a critical factor in the control of cardiac hypertrophy (de Bold *et al.* 2001), few studies have investigated this action during pregnancy.

The purpose of this study was, therefore, to investigate the expression of natriuretic peptides, NOS and cGMP in the rat LV throughout gestation and early postpartum. Because of their contribution to ANP and NO release in the cardiovascular system, estrogen and oxytocin receptors (OTR) were also evaluated (Gutkowska *et al.* 1999, Jankowski *et al.* 2001, Mukaddam-Daher *et al.* 2001, Wang *et al.* 2003). Our results demonstrate that cGMP concentration in the rat LV is controlled by both NOS and natriuretic peptides, but their inverse expressions at different stages of gestation suggest distinct roles in the control of physiologic cardiac hypertrophy.

Materials and Methods

Animals

These experiments were performed in accordance with the Guidelines of the Canadian Council on Animal Care, after approval of the animal care committee of the Centre hospitalier de l'Université de Montréal. Female Sprague–Dawley rats purchased from Charles River (St Constant, QC, Canada) were housed in a temperature- and light-controlled room with food and water available *ad libitum*. Age-matched virgin and pregnant animals were weighed and killed by decapitation at days 7, 14 or 21 (term) of gestation, and at days 1 or 4 postpartum. Hearts were quickly removed, dissected, and blotted dry. Heart atria and ventricles were separately weighed, and then frozen in liquid nitrogen, and stored at −80 °C for RNA or protein extraction.

Ventricular cGMP assay

Measurement of cGMP concentration has already been described (Gutkowska *et al.* 1999). Briefly, the dissected tissues were cut into small pieces and homogenized in 5 vol of ice-cold 5% (v/v) trichloroacetic acid (TCA). The homogenates were centrifuged at 600 ***g***, for 5 min, at 4 °C. TCA was extracted from the reaction mixture with 1:1 triocytydylamine and 1,1,2-trichlorofluoroethane (Sigma). cGMP concentration of the aqueous phase was quantified by radioimmunoassay, as described previously (Gutkowska *et al.* 1999), and expressed as picomoles per wet tissue weight in grams. The intra- and interassay coefficients of variation of this assay were 8% and 15% respectively.

Northern blot analysis

RNA was extracted from the LV with Trizol (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's instructions. Total RNA was subjected to electrophoresis on 1·5% agarose gels containing 0·22 M formaldehyde and transferred onto nylon membranes (Hybond N+; Amersham) by capillary blotting. Immobilized RNA samples were hybridized with randomly primed ^{32}P-cDNA probes corresponding to ANP and alpha-tubulin mRNA sequences. The probes were labeled with a random priming kit (Gibco BRL, Bethesda, MD, USA) and ^{32}P-dCTP (3000 Ci/mmol; Amersham). The Pst 1-digested 660-bp fragment from plasmid clone PN-1–11 served as the ANP probe (a gift from Dr Mona Nemer, Institut de Recherches Cliniques de Montréal, Montreal, QC, Canada).

Complementary DNA synthesis and semiquantitative polymerase chain reaction (PCR)

Total RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (200 U; Life Technologies), with random primers (200 ng) in a 20 µl reaction mixture. To compare different expression levels, semiquantitative PCR was performed. A volume of 10 µl first-strand cDNA was added to a PCR mixture and amplified for 25–35 cycles by incubation at 95 °C for 1 min, at 57–65 °C for 1 min, and at 72 °C for 1·5 min, with a final incubation at 72 °C for 3 min, all in a Robocycler gradient 40 thermocycler (Stratagene, La Jolla, CA, USA). The RT reaction products were subjected to PCR amplification with the following primers:

- ANP forward, 5′-CAGCATGGGCTCCTTCTC CA-3′

- ANP reverse, 5′-GTCAATCCTACCCCCGAA GCAGCT-3′
- BNP forward, 5′-CCATCGCAGCTGCCTGGCC CATCACTTCTG-3′
- BNP reverse, 5′-GACTGCGCCGATCCGGTC-3′
- GC-A forward, 5′-AAGCTTATCTGGAGGAGA AGCGCA-3′
- GC-A reverse, 5′-TCAGCCTCGAGTGCTACA TCCCCG-3′
- GC-B forward, 5′-GCTACATGGTACCACCAT ATTTGGACAACCTC-3′
- GC-B reverse, 5′-CAGGAGTCCAGGAGGTCC TTTTCG-3′
- NPR-C forward, 5′-ATCGTGCGCCACATCCA GGCCAGT-3′
- NPR-C reverse, 5′-TCCAAAGTAATCACCAAT AACCTCCTGGGTACCCGC-3′.

The specificity of the amplified products was verified by sequencing. Amplification of 18S RNA, used as an internal standard, followed the manufacturer's protocol (Ambion, Austin, TX, USA). PCR products were fractionated onto 1·2% agarose gels containing ethidium bromide. The signal intensities were measured in at least eight individual animals with two independent reverse transcriptions (RT) per sample. The intensity of the bands was determined by densitometry with Molecular Imaging Software (Image-Quant Software, Molecular Dynamics, Sunnyvale, CA, USA). To validate this RT-PCR assay as a tool for the semiquantitative measurement of mRNA, dose–response curves were established for different amounts of total RNA extracted from the rat LV, and the samples were quantified in the curvilinear phase of PCR amplification. The expression levels were normalized against 18S or GAPDH. No difference was observed in 18S or GAPDH levels at any stage.

Western blot analysis

Western blot analysis was conducted as described elsewhere (Wang *et al*. 2003). Proteins were separated on an 8% sodium dodecyl sulfate gel and electrotransferred to nitrocellulose membranes (Hybond-C; Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada). Unbound sites were blocked overnight at 4° C with 10% (w/v) nonfat milk in Tris–buffered saline containing 20 mmol/l Tris–HCl (pH 8·0), 140 mmol/l NaCl and 0·05% (w/v) Tween-20. The membranes were then probed with mouse monoclonal antibodies for iNOS or eNOS (BD Biosciences, San Jose, CA, USA). OTR were investigated with rabbit polyclonal antibodies (generous gift from Dr Kate Whittington, University of Bristol, UK), and estrogen receptors using rabbit polyclonal antibody (Research Diagnostics, Pleasant Hill, NJ, USA). The membranes were washed and incubated with secondary antibodies linked to horseradish peroxidase-labeled antimouse or antirabbit immunoglobulin G (Amersham Pharmacia Biotech) for 2 h. The blots were developed in an enhanced chemiluminescence system (Amersham Pharmacia Biotech) and visualized by exposure to Kodak radiographic film. The accuracy of protein loading on the gel was verified by reprobing with rabbit β-actin antibody and protein coloration on the membranes. Density of bands was measured with the Scion program (NIH, Bethesda, MD, USA).

Immunocytochemistry (ICC)

Prior to ICC, OTR and ER antigenic sites were retrieved by immersing the sections in citrate buffer 0·1 M, pH 6·0, heated to 90 °C for 20 min and allowed to cool slowly to room temperature. For incubation, primary antibodies diluted 1/300 in blocking solution (No. 00–8020; Zymed Laboratories, San Francisco, CA, USA) were revealed by the biotin–streptavidin method (No. 95–999, Histostain-DS; Zymed Laboratories) (Jankowski *et al*. 2004). Controls obtained by the omission of primary antibodies were negative, emphasizing the specificity of ICC.

Statistical analysis

The data are expressed as mean ± S.E.M. Normalized data of nonpregnant, pregnant and postpartum groups were compared by ANOVA, followed by Dunnett's test or, in the case of comparison with only one group, by a two-tailed version of Student's *t*-test. $P<0·05$ was accepted as the level of significance. For calculations, Prism 3 program was applied (GraphPad Software, San Diego CA, USA).

Results

Changes in body and heart weights during rat gestation

Table 1 shows that, compared with nonpregnant controls, rat body weight increased on gestational days 14 (223 ± 3 to 264 ± 6 g or 118%; $P<0·05$) and 21 (412 ± 8 g, 184%; $P<0·001$). LV mass increased in a similar manner. Figure 1 shows a positive correlation between LV weight and body weight. On gestational day 14, mean LV weight was 755 mg (112% of nonpregnant levels), and was further increased to 866 mg (128%) on day 21. As shown in Table 1, body weight remained above the control value on days 1 and 4 postpartum (127% and 132% respectively), and was associated with increments in ventricular weight (127% and 123%; $P<0·05$). A positive correlation ($r=0·81$ and 0·73, respectively) between LV weight and body weight was found.

Atrial weight was not significantly altered and, on day 21 of gestation, represented 97·5% of nonpregnant control levels. The elevation of atrial weight started in the postpartum period, and was significantly higher (118%) on day 4 than in the nonpregnant controls.

Table 1 Body weight (BW) and heart weight in rats at different days of gestation and early postpartum

		Gestation (in days)			**Postpartum** (in days)	
	Nonpregnant	7	14	21	1	4
Body weight (g)	223 ± 3	216 ± 5	264 ± 6*	412 ± 8*	284 ± 14*	296 ± 14*
Heart (mg)	712 ± 22	709 ± 13	799 ± 29	914 ± 42*	898 ± 38*	866 ± 44*
H/BW (mg/g)	3·2 ± 0·1	3·3 ± 0·1	3·0 ± 0·1	2·2 ± 0·1*	3·2 ± 0·1	2·9 ± 0·1
Ventricles (mg)	663 ± 22	665 ± 13	755 ± 29*	866 ± 42*	842 ± 37*	808 ± 54
V/BW (mg/g)	3·0 ± 0·1	3·1 ± 0·1	2·9 ± 0·1	2·1 ± 0·1*	3·0 ± 0·1	2·8 ± 0·1
Atria (mg)	49 ± 2	44 ± 1	44 ± 1	48 ± 1	55 ± 4	58 ± 4*
A/BW (mg/g)	0·22 ± 0·01	0·20 ± 0·01	0·17 ± 0·01*	0·12 ± 0·01*	0·19 ± 0·01	0·20 ± 0·02

Values are mean ± S.E.M. $n=8$. *$P<0.05$ vs non pregnant.
H: heart; V: Ventricles; A: Atria.

cGMP concentration in the LV of nonpregnant and pregnant rats

Compared with nonpregnant rats, cGMP concentration was highest in the LV on day 14 of gestation (2·98 ± 0·05 vs 4·65 ± 0·17 pmol/g wet weight) and then decreased at term to 2·32 ± 0·10 pmol/g. cGMP concentration in the LV was elevated on postpartum days 1 and 4 (6·01 ± 0·15 and 9·21 ± 1·79 pmol/g respectively) (Fig. 2).

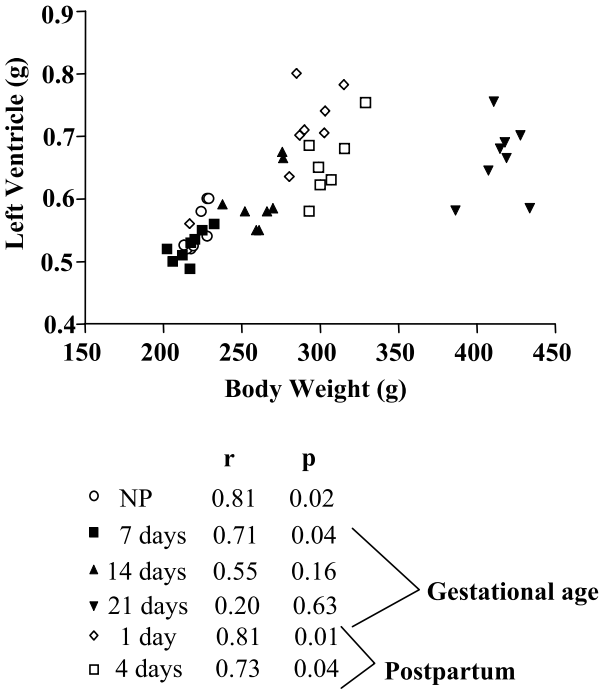

Figure 1 Correlation of changes in left ventricular weight and body weight on different days of rat gestation and early postpartum ($n=8$, each group; r=Pearson correlation coefficient, P=significance value).

Western blot analysis of eNOS, iNOS, ER and OTR

In parallel with gestation-associated changes in LV cGMP levels, changes in OTR, iNOS and eNOS proteins were observed. Figure 3 shows that OTR (67 kDa)-, iNOS (135 kDa)- and eNOS (140 kDa)-specific proteins in the LV were elevated on days 7 and 14 of pregnancy as compared with nonpregnant controls. However, at term, the expression of these proteins was decreased three to four times below LV levels in nonpregnant controls, and then increased after parturition, to exceed control values on postpartum day 4. As presented in Fig. 4, the ERα were slightly higher on gestational day 14 (35%), but were below control level (–60%) at postpartum. Western blot analysis detected no changes in ERβ during pregnancy and postpartum.

Figure 5 shows immunolocalization of OTR around the uterine lumen, within epithelial cells (small arrow), uterine glands (medium arrow) and myometrial smooth

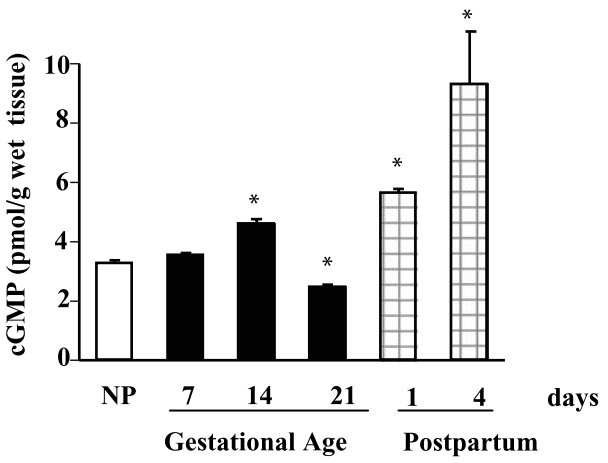

Figure 2 cGMP concentration in left ventricles of nonpregnant (NP), and pregnant rats at different stages of gestation and postpartum rats ($n=5$–6 rats per group). Values are mean ± S.E.M. *$P<0.05$ vs NP.

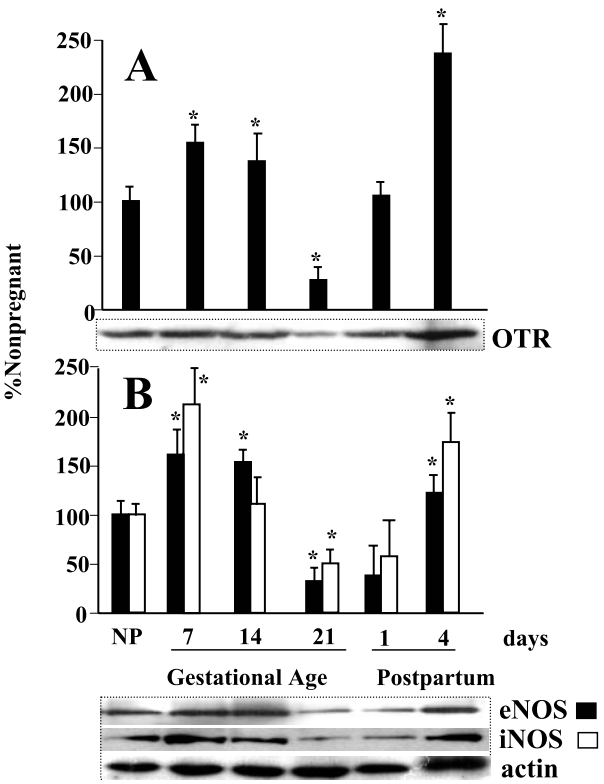

Figure 3 Western blot analysis of (A) oxytocin receptors (OTR), and (B) endothelial (eNOS) and inducible (iNOS) nitric oxide synthases in left ventricles of rats during gestation and early postpartum. Representative immunoblot shows OTR (67 kDa), eNOS (140 kDa) and iNOS (135 kDa). Band optical density was adjusted to the corresponding actin band and expressed as a percentage of the nonpregnant (NP) value obtained in the same experiment. Three independent experiments gave similar results (*P<0·05 vs NP).

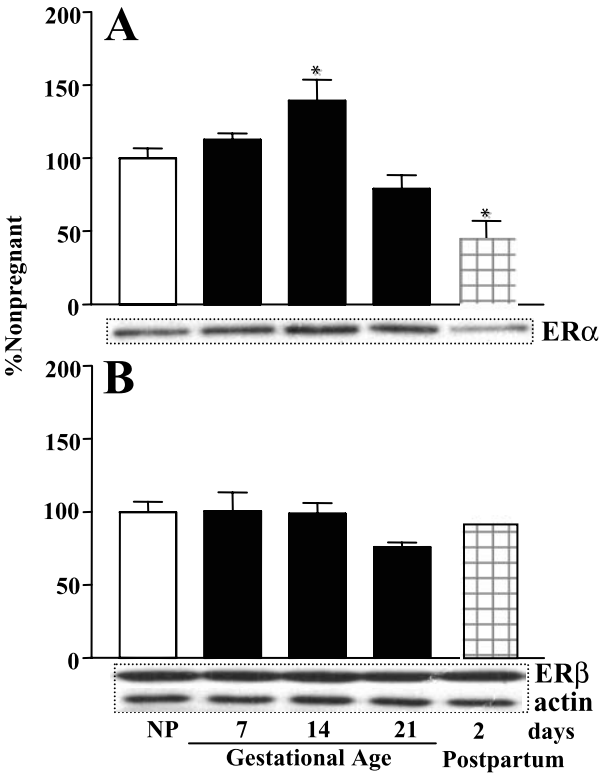

Figure 4 Western blot analysis of estrogen receptors α (ERα) (A) and β (ERβ) (B) in left ventricles of rats during gestation and early postpartum. Representative immunoblot shows ERα (62 kDa) and ERβ (55 kDa). Band optical density was adjusted to the corresponding actin band and expressed as a percentage of the nonpregnant (NP) value obtained in the same experiment. (*P<0·05 vs NP).

muscles (large arrow). ERα immunoreactivity was also detected in cell nuclei of atrial cardiomyocytes (arrows in Fig. 5C). Much lower ERα immunoreactivity could be detected in the heart ventricle (Fig. 5D). These results, confirming already known sites of OTR and ERα expression, indicate antibody specificity. In addition, no ERβ staining was observed in the rat heart sections.

Expression of natriuretic peptides

ANP mRNA expression in LV was not altered throughout gestation (Fig. 6A), but significantly increased postpartum. The results obtained by RT-PCR confirmed the Northern blot analysis and showed twofold increase in ANP mRNA in the LV of postpartum rats (Fig. 6B). Similarly, RT-PCR analysis revealed that BNP mRNA was unchanged on day 7, and then decreased to 60% by day 14, and further to 10% at term, as compared with nonpregnant controls considered as 100% (Fig. 6C). In the early postpartum period, LV BNP mRNA returned to control levels.

Expression of natriuretic peptide receptors

Figure 7 presents the results of RT-PCR analysis of GC-A, GC-B, and NPR-C mRNA expression in LV of nonpregnant and pregnant rats in different stages of gestation and postpartum. Notably, compared with nonpregnant controls, GC-A mRNA, transducing ANP and BNP signals, was downregulated on days 14 and 21 of pregnancy, and in the early postpartum period (Fig. 7A). GC-B mRNA was also significantly lower in the LV at day 21 and postpartum than in nonpregnant controls (Fig. 7B). In contrast, NPR-C mRNA responsible for natriuretic peptide clearance (Fig. 7C) was significantly higher on day 21 of gestation.

Discussion

The present study demonstrates that LV weight positively correlates with body weight gain during early gestation

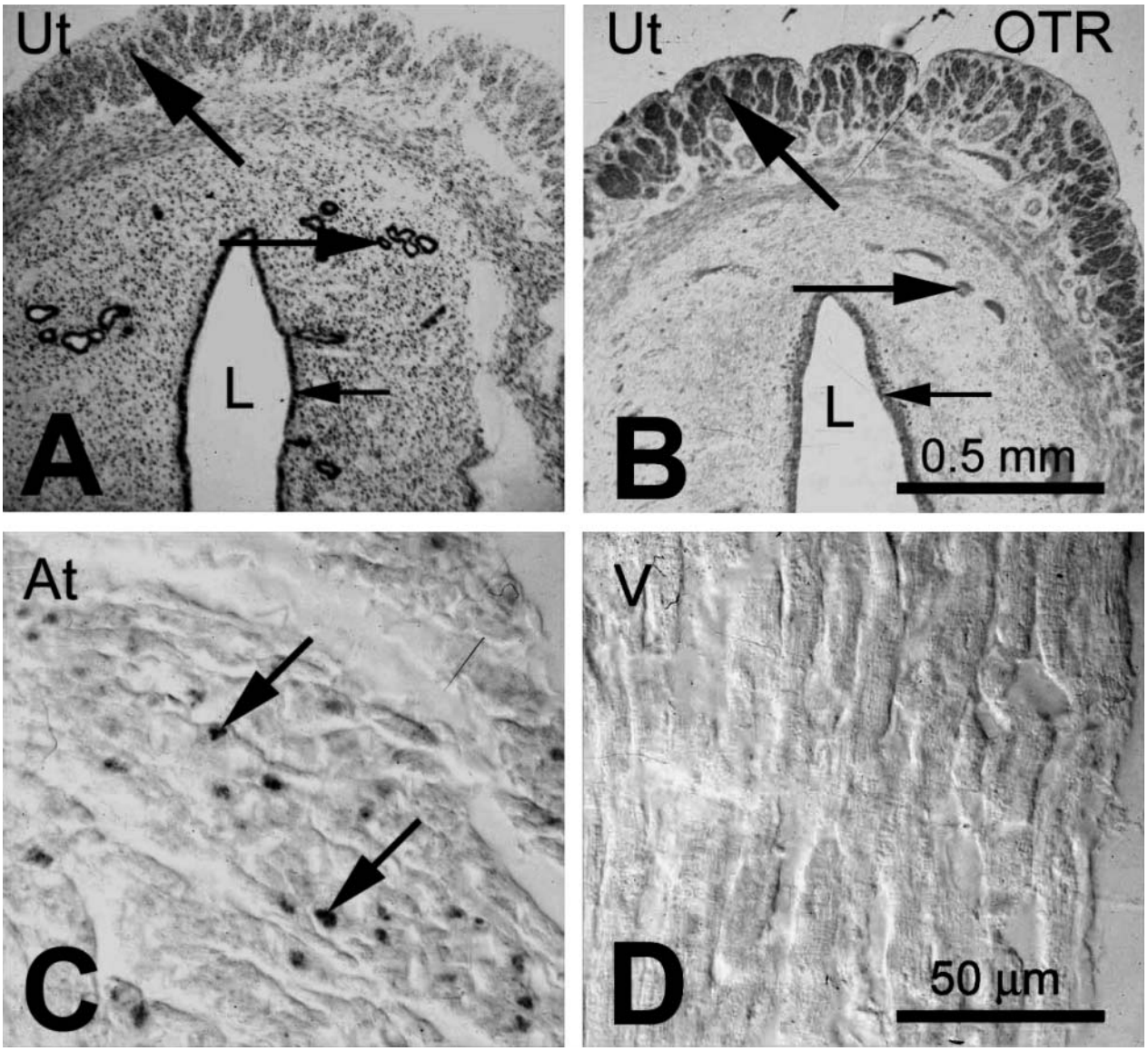

Figure 5 Immunocytochemical localization of ERα (A, C and D) in the rat uterus (Ut) and heart atrium (At) and ventricle (V). (B) Comparable immunocytochemical staining for oxytocin receptor (OTR) in the uterus is shown around the uterine lumen (L), and within epithelial cells (small arrow), uterine glands (medium arrow) and myometrium smooth muscles (large arrow). Cell nuclei within numerous atrial cardiomyocytes displayed ERα immunoreactivity (arrows in C). Much less, if any, ERα immunoreactivity could be seen in the heart ventricle (D). Magnification (A and B) ×54; (C and D) ×540.

and postpartum, but not at term. LV cGMP increases by day 14 of pregnancy in parallel to elevations of eNOS, iNOS, ERα and OTR proteins, but not with the expression of ERβ, natriuretic peptides and their receptors. At term, LV cGMP decreases as does the expression of cardiac genes involved in particulate and soluble GC activities. These include natriuretic peptides, their GC receptors, eNOS, iNOS, and OTR. In contrast, the ventricular NPR-C transcript is elevated, suggesting that augmented cardiac natriuretic peptide clearance occurs at term. At postpartum, the cGMP-generating systems are activated, in parallel to a gradual decrease in cardiac hypertrophy.

It is established that cardiac adaptations occur in pregnancy. The LV increases in mass to preserve contractile function when cardiac workload is increased to accommodate pregnancy-associated plasma volume expansion (Churchill *et al.* 1980, Atherton *et al.* 1982, Barron 1987). Vascular and cardiac adaptations during pregnancy are influenced by reproductive hormones. The actions of estrogens and progesterone are mediated by specific

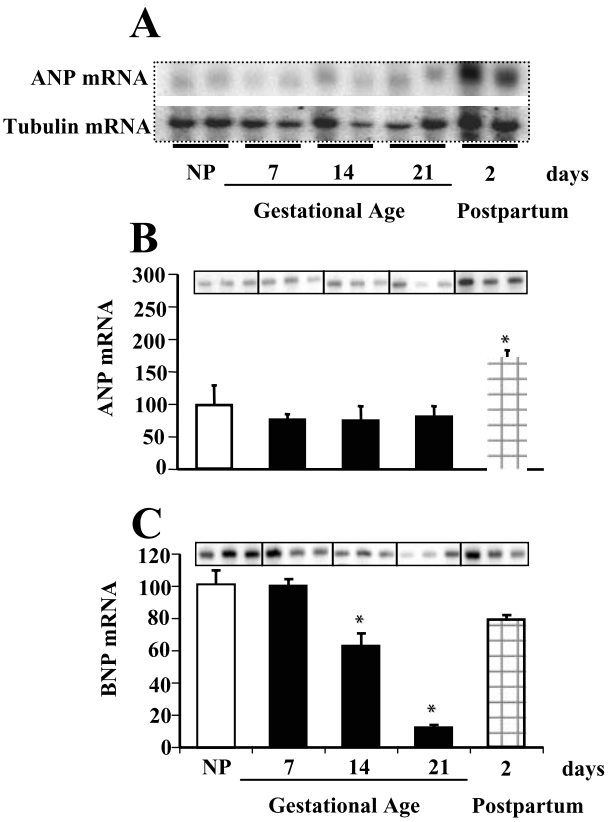

Figure 6 Effect of rat gestation and early postpartum on atrial natriuretic peptide (ANP) expression in left ventricles as demonstrated by Northern blot (A) and RT-PCR (B). RT-PCR was also used for semiquantitative measure of BNP mRNA (C). The RT-PCR products were separated by electrophoresis in 1·5% agarose, and then quantified by PhosphorImager software (Molecular Dynamics), and the results were adjusted as the ratio of the signal given by bands of ANP or BNP to the corresponding 18S band. Values are mean ± S.E.M. adjusted to nonpregnant (NP) values (100%). $n=6$ each; $^*P<0{\cdot}05$ vs NP.

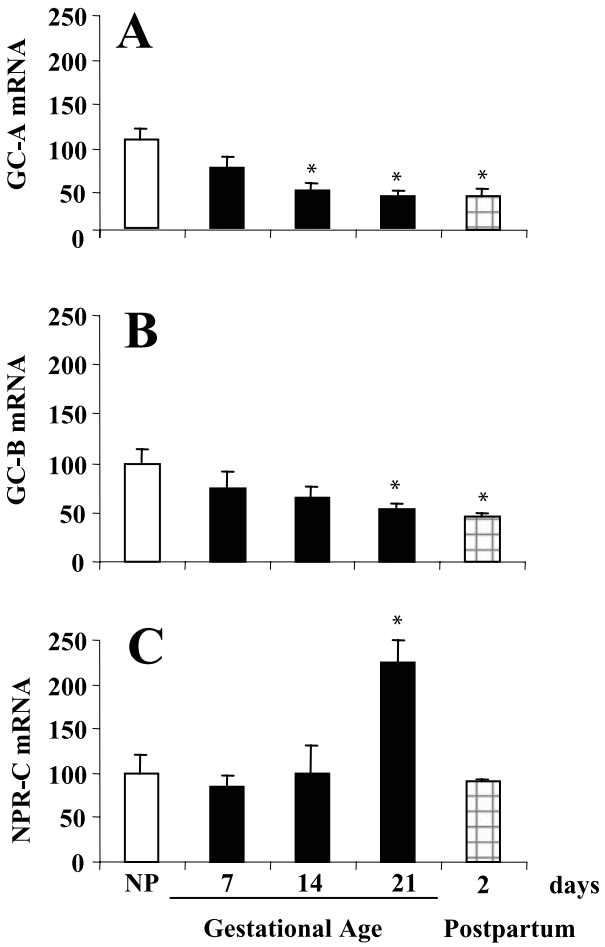

Figure 7 Relative quantification of natriuretic peptide receptor mRNA in left ventricle of rats during gestation and early postpartum by RT-PCR. The separated RT-PCR products were quantified by PhosphorImager software, and the results were adjusted as the ratio of the signal given by bands of GC-A (A), GC-B (B) and NPR-C (C) to the corresponding 18S band. Values are mean ± S.E.M. adjusted to nonpregnant (NP) values (100%). $n=6$ each; $^*P<0{\cdot}05$ vs NP.

binding sites in vascular tissue and in the heart. Their hormonal effects include changes in the synthesis or release of cardiac natriuretic peptides, and in the oxytocin system (Mukaddam-Daher *et al.* 2002, Wang *et al.* 2003) as well as NOS and cGMP regulation (Weiner *et al.* 1994, Linke *et al.* 2002). In addition, direct interaction between these systems may also be involved in pregnancy-associated cardiac remodeling.

Parallel changes of the expression of LV NOS and OTR were observed during gestation and postpartum. These findings are consistent with OTR influencing NO activity, as previously demonstrated in the dog heart (Mukaddam-Daher *et al.* 2001), and with the predominant expression of OTR in endothelial cells of coronary arteries, providing a morphologic basis for eNOS and OTR interactions in the heart chambers (Jankowski *et al.* 2004).

An earlier report indicates that eNOS is transiently elevated during pregnancy in rats, and the increase is associated with enhanced NO-dependent control of myocardial oxygen consumption at a time when cardiac output is augmented (Linke *et al.* 2002). Despite a significant increase in eNOS protein expression at day 7 of pregnancy, the investigators observed a nonsignificant trend toward increased myocardial oxygen consumption. Similarly, in the present study, at day 7 of gestation, LV cGMP tended only to increase, despite the significant increases in eNOS proteins. There is no explanation for this lack of parallelism between eNOS and cGMP production. A delay in pregnancy-associated eNOS phosphorylation is a plausible explanation that remains to be proven.

Interestingly, we found that cGMP increased in the LV at day 14 of gestation in parallel with increments of eNOS and OTR, but low expression of GC-A and

GC-B mRNAs. Downregulation of cardiac natriuretic peptide receptors in LV during pregnancy may be physiologically required to control the negative inotropic activity of natriuretic peptides and to allow the development of pregnancy-induced LV hypertrophy. The mechanism of low expression of GC-A and GC-B mRNAs in this period is not known; however, it may be explained by two hypotheses. First, alterations of female steroids during pregnancy can downregulate natriuretic peptide receptors in several organs (Vaillancourt *et al.* 1997, 1998). The presence of ERs in the isolated cardiac myocytes and fibroblasts (Grohe *et al.* 1997) and the fact that these cells contain functional natriuretic peptide receptors suggest that ERs may be involved in pregnancy-mediated changes in the heart. The present ICC data indicate that the density of ERs receptors is much lower in cardiac ventricles than in atria. However, these receptors are functional and stimulate ANP expression and cGMP production in cultured rat cardiomyocytes (Babiker *et al.* 2004). We have demonstrated that ERα, but not ERβ, changes during pregnancy. These ERα receptors are, to some extent, upregulated at midgestation in association with similar cGMP elevation. We have already demonstrated that ovarian natriuretic peptide receptors may be a target of estrogen action (Jankowski *et al.* 1997, Gutkowska *et al.* 1999). A similar effect in the heart may be proposed where natriuretic peptide receptors exert their physiologic effect on both myocytes and nonmyocytes (Cao & Gardner 1995), and where natriuretic peptide-signaling events have been detected in both cell types. Second, it has already been shown that downregulation at the level of GC-A transcription appears to depend on intracellular increases of cGMP, and not on the natriuretic peptide ligand itself (Cao & Gardner 1995). Functional analysis of the GC-A promoter has demonstrated that long-term negative regulation of GC-A occurs via a putative cGMP-responsive element located at more than 1 kbp upstream of the transcription start site (Roubert *et al.* 1987, Tremblay *et al.* 2002).

The importance of cGMP in the control of transient LV hypertrophy and the changes in contractility during pregnancy remain to be clarified. Evidence from animal and human studies suggest that the cGMP signaling pathway is gestationally regulated (Weiner *et al.* 1994, Linke *et al.* 2002). We observed that, at term, LVcGMP declined below nonpregnant control levels, confirming the previous report by Weiner *et al.* (1994). This decrease in cGMP may have resulted from downregulated natriuretic peptides and their GC receptors, as well as upregulated clearance receptors, which act as a buffering system, reducing the effective pool of natriuretic peptides. Decline of LV OTR, eNOS, iNOS and ERα, and, as we have reported previously, plasma ANP (Mukaddam-Daher *et al.* 2002) may also contribute to cGMP reduction in the LV. The reduced levels of cGMP at term favor a shift of balance toward enhanced heart work at the onset of labor. Therefore, cGMP may play an important role in maintaining cardiac quiescence during the final period of pregnancy, and a decrease in cGMP at term may contribute to the increase in heart rate and contractility during active labor.

Finally, an important and still unresolved issue is whether cGMP produced by *particulate* versus *soluble* GC acts differently inside the cells. For instance, the increase in cGMP mediated by ANP, but not by NO, prevents cardiomyocyte hypertrophy (Horio *et al.* 2000). It has also been shown that particulate GC-A, but not soluble GC, has potent effects on plasma membrane control of the calcium ATPase pump (Zolle *et al.* 2000). A recent study has also demonstrated that ANP/GC-A, but not NO/soluble GC, stimulates the translocation of PKG I to the plasma membrane (Airhart *et al.* 2003). It is hypothesized that cGMP-mediated signaling is compartmentalized within cells targeted by NO and natriuretic peptides (Kuhn 2003).

In conclusion, these findings imply that during pregnancy, LVNOS and natriuretic peptide systems exert direct actions on the heart, and are thus involved in adaptation to pregnancy-associated volume alterations.

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