Mechanical Stretch Increases Brain Natriuretic Peptide Production and Secretion in the Human Fetal Membranes

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Jorge A. Carvajal, MD, PhD¹, Ana M. Delpiano, MSc¹,
Mauricio A. Cuello, MD¹, and José A. Poblete, MD¹

Abstract

Brain natriuretic peptide (BNP) is synthesized by human fetal membranes, both the amnion and chorion. This locally produced BNP inhibits the contraction of the human myometrium, contributing to the maintenance of myometrial quiescence during pregnancy. We tested the hypothesis that BNP production is increased by fetal membrane stretching, which is predicted to occur in the expanding uterus, and inhibited by epidermal growth factor (EGF), whose production in the fetal membranes increases in late pregnancy. Term fetal membranes were obtained during elective cesarean delivery before labor. Sections of membranes were placed in an isolated chamber containing DMEM: F12 medium (37°C) and stretched with a 35 g weight. Medium and tissue samples were collected at 0, 3, 6, 18, and 24 hours for measurement of messenger RNA (mRNA) and BNP levels in the presence/absence of EGF (2×10^{-9} mol/L). Inducible nitric oxide synthase (iNOS) and β -actin were also evaluated to discard a nonspecific effect of mechanical stretch on protein expression. We found that amnion and chorion stretching increased the BNP mRNA (reverse transcription–polymerase chain reaction [RT-PCR]) and protein (radioimmunosorbent assay [RIA]) levels from 18 hours onward. The effect of stretching was inhibited by EGF (2×10^{-9} mol/L). Stretch did not increase iNOS or β -actin protein levels. We concluded that chorion and amnion stretching may increase BNP expression in the fetal membranes during pregnancy, while increasing biological activity of EGF may decrease BNP production in the chorion and amnion late in pregnancy. We postulate BNP is an important regulator of myometrial contractility during pregnancy, and its production is modulated by both stretch and progressive increase in EGF levels during pregnancy.

Keywords

fetal membranes, brain natriuretic peptide, pregnancy, women/humans, myometrial quiescence, mechanical stretching

Introduction

The precise regulation of gestational length is crucial for a healthy neonatal outcome. The myometrium is held for most of gestation in a physiological state of active relaxation, generally referred as *myometrial quiescence*.^{1,2} Myometrial quiescence is characterized by a profound refractoriness of the myometrium to substances that normally stimulate myometrial contractions. The mechanisms underlying the process of myometrial quiescence are poorly defined, although many mediators are proposed to play a role in this highly regulated process.¹

We previously hypothesized that the fetal membranes produce one or more factors that act in a paracrine fashion to inhibit myometrial contractions and thus are central to the maintenance of myometrial quiescence.³⁻⁵ We postulated that a locally produced natriuretic peptide may be involved in the control of myometrial quiescence because brain natriuretic peptide (BNP) was detected in human amniotic fluid and cultured amniotic cells⁶; and the presence of biologically active

types A and B natriuretic peptide receptors (particulate guanylate cyclase) was detected in the human myometrium.⁷

We previously reported in the myometrium of pregnant guinea pigs that among natriuretic peptides, BNP (compared to Atrial Natriuretic Peptide [ANP] and C-Type Natriuretic peptide [CNP]) was the most effective in inhibiting myometrial contractility in vitro.⁸ Furthermore, we demonstrated that the BNP-induced inhibition of myometrial contractility proceeded via a novel mechanism that did not involve the activation of either particulate guanylate cyclase or the cyclic guanosine monophosphate (cGMP) pathway.⁸

¹ Unidad de Medicina Materno Fetal, División de Obstetricia y Ginecología, Pontificia Universidad Católica de Chile, Santiago, Chile

Corresponding Author:

Jorge A. Carvajal, Marcoleta 391, Centro de Investigaciones Médicas, Pontificia Universidad Católica de Chile, Santiago, Chile.
Email: jcarva@med.puc.cl

Figure 1. Schematic drawing showing the acrylic chamber used to stretch the fetal membranes. The chamber was maintained in a 95% humidified incubator at 37°C with 5% CO₂. The 2 ends of the submerged membrane were attached to a nondistensible thread. The thread passes through a hole in the bottom of the middle wall, creating a pulley device. Stretch was applied by hanging 35 g from each end to generate a force of 0.343 N. The pH was measured every 6 hours and maintained at 7.42; if altered, the medium was replaced by fresh DMEM:F12 (37°C).

We recently reported, using human samples, that BNP (but not ANP or CNP) inhibited contractions of preterm, but not term, human myometrium.⁹ The BNP (both protein and messenger RNA [mRNA]) was detected in human fetal membranes (explants and cultured cells).⁹ The BNP level was higher in samples from preterm women not in labor than in those at term not in labor. Additionally, the BNP concentrations were significantly lower in women in spontaneous preterm labor.⁹ We therefore concluded that locally produced BNP may be involved in the establishment of myometrial quiescence during pregnancy. Further, a premature decrease in BNP production may cause preterm labor.⁹ Currently, it is not known how BNP production or release is regulated in human fetal membranes.

In cardiomyocytes both *in vivo*¹⁰⁻¹² and *in vitro*,¹³ the BNP levels (protein and mRNA) are increased by mechanical stretching. However, the effect of stretching on BNP expression has not been tested in noncardiovascular tissues. In the present investigation, we sought to test our hypothesis that BNP production is increased by fetal membrane stretching, which is predicted to occur in the expanding uterus. We also postulate that at the end of pregnancy, BNP production is

inhibited by epidermal growth factor (EGF), whose production in the fetal membranes increases in late pregnancy.

Material and Methods

Tissue Samples

The study protocol was approved by the Institutional Review Board. Women were included only after providing informed consent. Placentas were obtained prior to the onset of labor from women undergoing elective cesarean delivery at term for obstetric indications (eg, breech presentation, previous cesarean section). Fetal membranes (chorion and amnion) were excised. The amnion was carefully separated from the chorio-decidual membrane by gentle traction, and the decidua was scraped from the chorion. The chorion and amnion were separately maintained at 4°C in oxygenated Krebs buffer until used later the same day.

Isolated Membrane Chamber Preparation

To stretch amnion and chorion samples, the amnion and chorion derived from the same patient were cut into 6 cm × 2 cm pieces.

Each piece of the fetal membrane (approximately 500 mg) was placed in a specially designed double-wall acrylic chamber (Figure 1) containing 10 mL of 1:1 mixture of Dulbecco Modified Eagle Medium (DMEM) and Ham F-12 Nutrient Mixture (DMEM:F12; Sigma-Aldrich, St Louis, Missouri, Catalog # D 6421). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer is included in the medium at a final concentration of 15 mmol/L to add buffering capacity. The chamber was maintained in a humidified incubator at 37°C with 5% CO₂. The pH was measured every 6 hours and maintained at 7.42; if altered, the medium was replaced by fresh DMEM:F12 (37°C). The 2 ends of the submerged membrane were attached to a nondistensible thread, and stretch was applied by hanging a lead cube (35 g weight) from each end to generate a force of 0.343 N. The setup created a pulley device with the thread at the chamber wall (Figure 1). In exploratory experiments, we used different lead cubes. Hanging a lead cube larger than 50 g resulted in the rupture of the tissue, whereas a weight lower than 25 g did not produce changes in BNP expression (data not shown). Control membranes were kept in separate chambers without tension.

Stretching has been used to study BNP expression in cardiovascular tissues and cells, using perfusion systems or vacuum to deform a plastic petri dish. These studies have used both static and cyclic stimuli, with similar results.^{12,13} A continuous (noncyclic) stretch was used as the experimental stimulus to simulate the enlarging uterus during pregnancy.

Control and stretched amnion and chorion samples were snap frozen after 0, 3, 6, 18, or 24 hours and stored at -80°C until assayed. A sample of the medium in the chamber was also collected at each time point and stored at -80°C.

Effect of EGF on BNP Production

To our knowledge, only one group has studied BNP regulation in human fetal membranes.¹⁴ They showed that EGF (range 2×10^{-11} to 2×10^{-8} mol/L) inhibited BNP production.

To test the effect of EGF on BNP production in our model, the membranes were incubated in the acrylic chamber with EGF (2×10^{-9} mol/L) for 20 minutes before starting the stretching or control conditions. The EGF concentration was maintained until the end of experiment. The BNP mRNA and protein levels were measured in the presence or absence of EGF.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from tissues (chorion and amnion) as described.¹⁵ Complementary DNA (cDNA) synthesis was performed using reverse transcriptase (RT), according to the manufacturer's protocol (Superscript II, Invitrogen). Reverse-transcribed cDNA was amplified in a 25-μL polymerase chain reaction (PCR) reaction containing 200 μmol/L deoxyribonucleotide triphosphates (dNTPs), 1 μmol/L each primer, 100 ng cDNA, 2.5 U Taq DNA polymerase (Invitrogen) and 2 mmol/L MgCl₂. After an initial denaturation step at 94°C for

4 minutes, 36 cycles of PCR were performed with denaturation at 94°C for 40 seconds, annealing at 64°C for 30 seconds and extension at 72°C for 30 seconds. Specific primers were designed based on the published cDNA sequence to amplify a 252-base pair segment of the *BNP* gene (synthesized by Bios Chile I.G.S.A.). The nucleotide sequence for the sense primer was 5'-ttcttgcacatctggttcttct-3', and the sequence for antisense primer was 5'-gcagggtgttagaggaccatt-3'. Primers were also designed for the constitutively expressed housekeeping gene human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control (680 bp). The optical density of the BNP band was measured and normalized to the GAPDH band. In some reactions, cDNA was omitted to provide a negative control. In preliminary studies, the presumed BNP band was cut out, and the gene product was eluted using the QIAEX II Gel Extraction Kit (QIAGEN, Bothell, Washington). The product was sequenced, and its identity was confirmed.

Measurement of BNP by Radioimmunosorbent Assay

We measured the BNP contents of control and stretched human chorion and amnion samples and their respective culture media. The BNP was measured as previously described.⁶ Briefly, a sample (100–200 mg) of chorion or amnion (or the medium) from a single patient was boiled (5 min) in 2 to 4 mL of 1 mol/L acetic acid containing 20 mmol/L HCl and then homogenized. The homogenate was centrifuged (15000g) for 30 minutes at 4°C, and the supernatant was aspirated. The BNP was extracted by filtering the supernatant through a Sep-Pack C18 cartridge (Peninsula Laboratories, Inc) and then eluted from the column with 60% acetonitrile in 1% trifluoroacetic acid (TFA). The eluate was evaporated, and the pellet was suspended in 300 μL of assay buffer. The BNP was measured using a commercially available kit according to the manufacturer's protocol (Peninsula Laboratories, a division of BACHEM, Pennsylvania). Membranes and medium were assayed in duplicate. The intra- and interassay variations were each less than 5%.

β-Actin and Inducible Nitric Oxide Synthase Measurement by Western Blot

To determine whether the effect of stretching was specific for BNP and not a general effect on all proteins, we measured the changes in the expression levels of a constitutive gene (β-actin) and an inducible gene (inducible nitric oxide synthase [*iNOS*]) in stretched and control human fetal membranes by Western blot.

Samples of membranes (50 μg of protein) were boiled (5 minutes, 90°C) in loading buffer, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide gel), and then electrotransferred to nitrocellulose membranes. The nitrocellulose membranes were blocked (2 hours) and then incubated with primary antibody (Santa Cruz Biotechnology, Santa Cruz, California) for 1.5 hours at

Figure 2. Representative RT-PCR gel showing increased BNP mRNA expression after the mechanical stretching of the amniotic membrane. RT-PCR indicates reverse transcriptase–polymerase chain reaction; BNP, brain natriuretic peptide; mRNA, messenger RNA.

room temperature (1:1000 dilution). The membranes were washed and incubated with horseradish peroxidase–conjugated antirabbit antibody (1:3000, for 1 hour). Immunoreactive bands were detected using the Western Lightning–enhanced chemiluminescence (ECL) plus method (PerkinElmer Inc). The optical density of the bands was measured for the quantitative analysis.

Statistical Analysis

All data sets were subject to a test of normalcy (Shapiro–Wilk test), and parametric and nonparametric tests applied where appropriate. Statistical comparisons between the 2 groups were performed with the Student *t* test. For multiple group comparisons, either 1-way analysis of variance (ANOVA) followed by a post hoc Student–Newman–Keuls test (parametric) or Kruskal–Wallis 1-way ANOVA on ranks followed by Dunn’s multiple comparison test (nonparametric) was used. A 2-tailed $P < .05$ was considered indicative of statistical significance.

The “n” values represent the patient numbers. Several pieces of amnion and chorion were cut from a single patient and kept in separate chambers (stretch or control, either with or without EGF) to maintain the desired schedule for the experiments. We have several chambers (22 chambers) maintained in the humidified incubator at the same time to create the time line of the membranes from the same woman. Thus, each time point corresponds to a different section of the same membrane (ie, the same patient). Data are presented as the mean + standard error.

Results

Expression of BNP mRNA

The BNP expression was demonstrated in the human amnion and chorion by RT-PCR (Figure 2 shows a representative PCR gel of stretched amnion). Under the control conditions (without stretching), the BNP mRNA levels remain unchanged after 24 hours. The amnion and chorion samples that were continuously stretched for at least 18 hours showed increased BNP mRNA levels compared to control (Figure 3). The effect of stretching was inhibited in the presence of EGF (Figure 3).

The BNP Protein Content in the Fetal Membranes and Medium

The BNP was present in membranes and medium of control and stretched samples. Under the control conditions, the BNP concentration, both in the chorion and in the amnion, remains unchanged after 24 hours, both in the membranes (Figure 4) and in the medium (Figure 5). Stretching significantly increases the BNP concentration in fetal membranes (Figure 4) or medium from 18 hour onward (Figure 5). In the presence of EGF, the BNP concentration did not show any significant variation (Figures 4 and 5).

β -Actin and iNOS Content in the Fetal Membranes

We detected β -actin and iNOS in both the chorion and the amnion. The expression of both proteins (β -actin and iNOS) did not exhibit any significant variation after stretching (Figure 6).

Discussion

We tested the hypothesis that stretching would enhance BNP expression in fetal membranes (chorion and amnion). In support of our working hypothesis, we demonstrated that more than 18 hours of membrane stretching increased the BNP levels (mRNA and protein), an effect that was inhibited by preincubation with EGF. We ruled out the possibility that stretch increases the expression of all proteins because the expression levels of a constitutive gene (β -actin) and an inducible gene (iNOS) did not change after stretching in our model.

The BNP production can be regulated by stretch as well as by endocrine/paracrine mediators.¹⁶ We postulate that during pregnancy, the enlarging intrauterine content may stretch the amnion and chorion, thereby increasing the BNP levels and allowing this peptide to be the primary regulator of myometrial quiescence, in a paracrine fashion. We also postulate that before labor, BNP production decreases,⁹ most likely due to the increased production of EGF that may block the cellular pathway mediating stretch-induced BNP production. It is a common observation that the uterus most distended are those that are most frequently associated with premature labor. We postulate that if the uterus is overdistended by the fetuses (multiple pregnancy) or the amniotic fluid (polyhydramnios), the direct distension of the myometrial fiber may overcome any quiescent mechanism, thus inducing uterine contractions and preterm labor.¹⁷

Two possible mechanisms of stretching-induced BNP production and secretion have been demonstrated.¹⁸ The first mechanism is a direct effect of cell stretching, implying a role for the extracellular matrix (fibronectin) and membrane selectins.^{19–21} The second mechanism is an autocrine/paracrine pathway, involving angiotensin II and endothelin 1,^{11,18–21} which activate the p38 Mitogen Activated Protein Kinase (MAPK) and Extracellular signal Regulated Kinase (ERK) pathway, which in turn activates the BNP promoter.^{19,21} This

Figure 3. Stretching increases the BNP mRNA levels (RT-PCR) in human fetal membranes (chorion and amnion). In the presence of EGF (2×10^{-9} mol/L), the BNP mRNA level did not change in response to stretching. Values are mean $\pm$ SE. * = $P < .05$ versus control and 0 hours; $n = 10$ at each point. RT-PCR indicates reverse transcriptase–polymerase chain reaction; BNP, brain natriuretic peptide; mRNA, messenger RNA; EGF, epidermal growth factor; OD, optical density; SE, standard error.

Figure 4. Stretching increases the BNP content in the human chorion and amnion (radioimmunosorbent assay [RIA]); this effect was inhibited by EGF (2×10^{-9} mol/L). Values are the mean $\pm$ SE. * = $P < .05$ versus control and 0 hours; $n = 10$ at each point. BNP indicates brain natriuretic peptide; EGF, epidermal growth factor; SE, standard error.

Figure 5. Stretching increases the BNP content in the medium in which the human chorion and amnion were stretched (radioimmunosorbent assay [RIA]). The (2×10^{-9} mol/L) blocked the increased BNP production. Values are the mean $\pm$ SE. * = $P < .05$ versus control and 0 hours; $n = 10$ at each point. BNP indicates brain natriuretic peptide; EGF, epidermal growth factor; SE, standard error.

Figure 6. Stretching did not increase either β -actin or iNOS expression in the human fetal membranes (Western blot). Values are the mean + SE of the optical density of iNOS and β -actin protein bands. * $P < .05$ versus 0 hours; $n = 5$ at each point. The insert presents a representative blot. iNOS indicates inducible nitric oxide synthase; SE, standard error.

activation depends on the interaction of the transcription factor nuclear factor (NF)- κ B with 3 shear stress response element (SSRE)-like structures in the proximal hBNP promoter.²² Although all these mechanisms were described in cardiovascular tissues we postulate that the same mechanisms may control stretch-induced BNP production in the fetal membranes.

The EGF has been postulated to play a paracrine role in the initiation of human parturition.²³⁻²⁶ The EGF receptor in human fetal membranes (chorion and amnion)^{23,24} and the EGF concentration in the amniotic fluid, increased at the end of a full-term human pregnancy.^{25,26} It was shown that EGF stimulates the expression of cyclooxygenase 2 (COX-2)²⁷ and prostaglandin production (both PGE₂ and PGF_{2 α}) by the fetal

membranes.²⁵ Thus, at term pregnancy EGF may be related to myometrial activation and initiation of labor through increased prostaglandin production by the fetal membranes.

In our model, EGF inhibited the effect of mechanical stretching on BNP production. Thus, it is possible that at the end of pregnancy, the increased biological activity of EGF on the fetal membranes may reduce BNP production, allowing myometrial activation and subsequently the initiation of labor. In primary culture of human amniotic cells, it was shown that EGF inhibited BNP production.¹⁴ The EGF may inhibit stretching-induced BNP upregulation in our model by downregulating integrins and selectins, the main mechanosensors of stretch.¹⁸⁻²¹ In a model of rat intestinal ischemia/reperfusion injury, it was demonstrated that

EGF significantly downregulated the expression of P and E selectins.²⁸ In addition, EGF hyperactivity in ovarian tumor cells decreased integrin protein expression.²⁹

We observed increased expression and secretion of BNP by the human fetal membranes during the period of myometrial quiescence,⁹ supporting a role for BNP in the establishment and maintenance of human myometrial quiescence. The results of this investigation provide a possible mechanism for BNP regulation in the human fetal membranes: stretching may be the stimulus for BNP production and secretion by the fetal membranes during myometrial quiescence. Later, at the end of pregnancy, EGF may inhibit the effect of stretching on BNP production, leading to decreased BNP production and the loss of myometrial quiescence.

Declaration of Conflicting Interests

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