Biphasic Effect of Relaxin, Inhibitable by a Collagenase Inhibitor, on the Strength of Human Fetal Membranes

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Abstract. *Background: Relaxin has been proposed as a hormone involved in the collagen remodeling of the utero-placental unit. Materials and Methods: Human fetal membrane explants were incubated with H1 or H2 relaxin for 48 hours and stretched until rupture in a materials testing machine. Co-incubation with a synthetic collagenase inhibitor was performed in order to examine whether the effects of relaxin could be inhibited. The effects on hydroxyproline and histology were evaluated. Results: Biomechanical testing showed that H2 relaxin induced a biphasic weakening of human fetal membranes, an effect that was abolished after co-incubation with a collagenase inhibitor. H1 relaxin produced no significant effects on the biomechanical properties. The effects of H2 relaxin on the biomechanical properties were, however, not followed by changes in the hydroxyproline concentration or the histology. Conclusion: H2 relaxin had an effect on human fetal membranes and this effect may be mediated through collagenases.*

The collagen concentration of human fetal membranes decreases with advancing gestational age (1) and it also decreases in women with Preterm Prelabor Rupture of Membranes (PPROM) (2-3). It seems, therefore, that the fetal membranes, like the cervix, undergo changes during pregnancy (4). It is possible that relaxin is involved in this maturation because serum relaxin concentrations are increased in the 18th (5) and the 30th (6) gestational week in women with subsequent preterm labor or PPROM. These clinical observations are consistent with a previous study that demonstrated a weakening of the human fetal membranes after incubation with relaxin (7). *In vitro* studies show that relaxin stimulation of amniotic and chorionic cells induce increased collagenolytic activity (8), and that relaxin induced an increase in interstitial collagenase (Matrix Metalloproteinase-1 or MMP-1), stromelysin (MMP-3) and gelatinase B (MMP-9) in intact human fetal membranes after 48-hour incubation (9-10).

A synthetic inhibitor of collagenase BB-250, (British Biotechnology Limited, Oxford, England) inhibits bone resorption and loss of hydroxyproline in mouse calvaries stimulated with PTH(11). BB-250 inhibits human interstitial collagenase (MMP-1), stromelysin (MMP-3), gelatinase A (MMP-2), gelatinase B (MMP-9) and matrilysin (MMP-7) (data from BBL).

Two forms of human relaxin (H1 and H2) have been demonstrated (12). H2 relaxin is mainly of ovarian origin and is associated with the above-mentioned effects. H1 relaxin m-RNA has been isolated from human fetal membranes (13), the prostate gland (14) and the heart (15), however, the peptide remains to be demonstrated in tissue. It is not known whether H1 relaxin has physiological effects or whether it is a vestigial hormone. H1 seems to have 20% of the affinity of H2 relaxin to the relaxin receptor (16).

The aims of the present study were: 1) to examine the effects of relaxin on the biomechanical properties of human fetal membranes over a wide concentration range, 2) to examine whether these effects can be visualized histologically or biochemically, 3) to examine whether BB-250 could inhibit the effects of relaxin, and 4) to evaluate, and compare with H2, the effect of H1 relaxin on human fetal membranes.

Materials and Methods

The local Scientific Ethics Committee of Aarhus County, Denmark approved this experiment, and written consent was obtained from all participating patients.

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Dose-response curves after 48 hours of incubation with H2 relaxin.

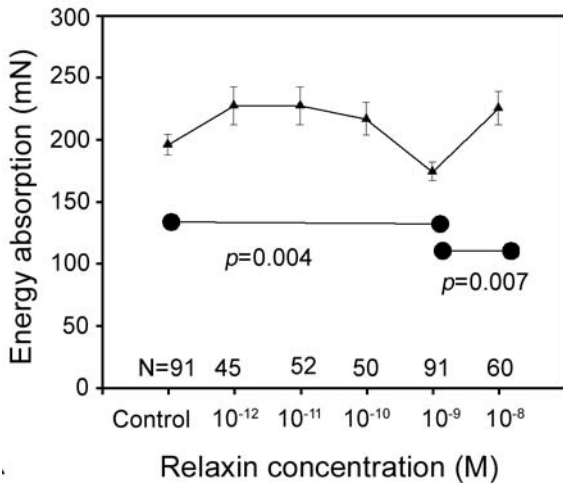
Figure 1. *Energy needed to rupture the amniotic membrane (N=91). Symbols depict mean and s.e.m. (Kruskal-Wallis, p=0.01).*

Figure 2. *The energy needed to rupture the fetal membranes after co-incubation with a synthetic collagenase inhibitor (BB-250). Incubation with relaxin (relative concentrations, nadir=1) caused a significant decrease in the energy absorption (Wilcoxon, p<0.01). This decrease was abolished by co-incubation with the collagenase inhibitor. Symbols indicate: control without collagenase inhibitor (black), relaxin without collagenase inhibitor (white), relaxin with collagenase inhibitor (white upslope pattern), control with collagenase inhibitor (black, checked pattern). Bars indicate mean and s.e.m.*

Tissue material. Fetal membranes (N=91) were taken within five minutes after delivery of the baby by elective Cesarean sections from term pregnancies before the onset of labor. The study was carried out from 1993-1997 at the Department of Obstetrics and Gynecology, Aarhus University Hospital, Denmark. The 91 specimens were used in 5 sub-studies (N=7, N=27, N=26, N=11, N=20).

Tissue incubations. Fetal membranes were cut from the placental edge and placed in ice-cold phosphate-buffered saline (PBS, pH 7.4). Samples were taken, as paired specimens, halfway between the placental edge and the incision. For blinding purposes, each specimen was assigned a randomly allocated number. The chorioamniotic specimens (3 x 3 cm) were incubated for 48 hours in M-199 (Earle's salts base with L-glutamine, Biological Industries, Israel). Incubations were done with and without human relaxins: H1 relaxin (a-chain 24, b-chain 32 amino acids, Professor G. Bryant-Greenwood, University of Hawaii, Honolulu, USA) and H2 relaxin (a-chain 24, b-chain 29 amino-acids, Genentech Inc. San Francisco, CA, USA) at different concentrations (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M). In addition, incubations were done with and without a synthetic collagenase inhibitor (10^{-6} M CI-1, N-[3-N-(benzyloxycarbonyl) amino-(R)-carboxypropyl]-L-leucyl-O-methyl-L-tyrosine N-methylamid by British Biotechnology Limited) (11). The IC_{50} specifications are as follows: MMP-1 5nM, MMP-3 20nM, MMP-2 20nM, MMP-9 2nM and MMP-7 10nM (Information from British Biotechnology Limited). Following 48-hour incubation, the saturation of both CO_2 and O_2 and the pH of the media were within the normal physiological range.

Biomechanical testing. Three strips (4 by 18mm) were punched out of each specimen (N=91+30+52+54+91+50+21=389) using a cutting instrument of razor blades in parallel. The biomechanical testing was undertaken using a materials testing machine (Alwetron TCT5, Lorentzen and Wettre, Stockholm, Sweden). The strips we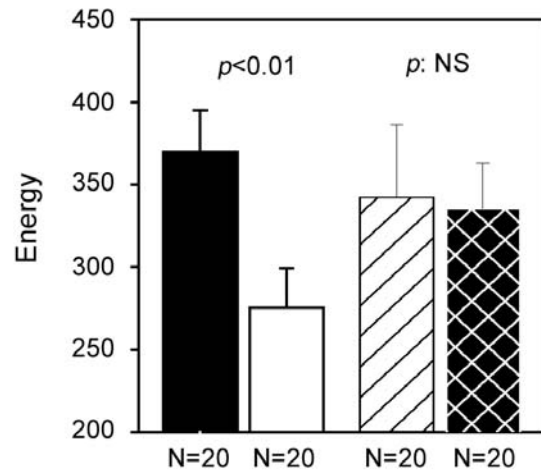re mounted in 2 clamps, the distance between which was increased at a constant speed of 10 mm/min until rupture of the tissue; load values were registered continuously. This method has been previously described in detail (7, 17).

Histology. Specimens, fixed in 3% v/v glutaric-aldehyde with 5% sucrose, were stained using Masson tricrome. Cleavage of the spongy layer and disorganization of the tissue (18) were evaluated blindly on three- and two-point scales, respectively.

Hydroxyproline quantification. The hydroxyproline concentrations were measured as described by Stegemann and Stalder (19).

Statistics. A mean value was calculated for each specimen from the 3 strips that were taken for biomechanical testing, and only this value was used for all further analyses. All the results are given as mean values ± standard deviation (SD). As the material does not follow a normal distribution, Kruskal-Wallis and then Wilcoxon or Mann-Whitney test were performed. Statistical significance in the two-sided analyses was taken as $p<0.05$.

Results

Biomechanical testing. The energy needed to rupture the amniotic membrane was significantly different between groups (Kruskal-Wallis, $p=0.01$, N=91). Specimens incubated with relaxin (10^{-9} M) were significantly weaker than their paired controls (Wilcoxon, $p=0.004$, N(pairs)=91)

and significantly weaker than their paired specimens incubated with 10^{-8} M (Wilcoxon, $p=0.007$, N(pairs)=60), Figure 1.

Collagenase inhibitor. BB-250 abolished the effect of relaxin on the biomechanical properties of human fetal membranes (Figure 2).

Histology. No significant differences were seen in the histological morphology after incubation with H2 relaxin for the disorganization of the tissue (NS, $p=1$, N=5), nor for the degree of cleavage in the spongy layer (NS, $p=0.7$, N=5).

Hydroxyproline. No statistically significant differences were found in the hydroxyproline concentration of the intact specimens after incubation with H2 relaxin (N=37). The controls had a hydroxyproline concentration of 29.6 mg/mg (SD=10.7) dry weight compared with 31.5 (SD=10.9) µg/mg after incubation with 10^{-9} M H2 relaxin.

H1 relaxin and biomechanical properties. H1 relaxin showed no significant effects on the biomechanical properties after 48 hours of incubation; however, a tendency for all variables to decrease was seen at 10^{-9} M, a decrease established with H2 relaxin. Thus, the energy needed to rupture the amnion decreased 5% after incubation with 10^{-9} M H1 relaxin (NS, $p=0.8$, N=10).

Discussion

The present study confirmed that human fetal membranes weaken after incubation with H2 relaxin at a concentration of 10^{-9} M (7). The biphasic concentration-response curve with no effect on fetal membranes at relaxin concentrations of 10^{-10} M or 10^{-8} M has not previously been published, but is well known in other human tissues (20, 21).

Co-incubation with a synthetic enzyme inhibitor abolished the relaxin-induced weakening of the fetal membranes. This enzyme inhibitor inhibits MMP-1, MMP-3, MMP-2, MMP-9 and MMP-7. This result is in line with previous results demonstrating that relaxin increases MMP-1 (10) and/or MMP-3 and MMP-9 (9) in fetal membranes. Moreover, MMP-9 activity in human fetal membranes increases with labor (22). Relaxin is known in other tissues to promote matrix remodeling *in vitro* (23-25).

The weakening of the fetal membranes after incubation with relaxin can, in part, explain the association between high serum H2 relaxin and increased risk of preterm delivery (5, 6, 26) or PPROM (5). The concentration of free relaxin in the well after adding 10^{-9} M of relaxin is equivalent to the serum concentration during pregnancy at term (7, 27). The biphasic concentration-response curve, however, precludes a simple interpretation of this association. Moreover, H2 relaxin is synthesized not only in the ovaries but also in the cytotrophoblasts and in the deciduas (28), and the local concentrations in the fetal membranes are therefore difficult to predict. We found increased data precision after scoring by the nadir of the paired concentration-response curve rather than using factual relaxin concentrations. This can be explained either by different sensitivity to relaxin among fetal membranes, or by problems in obtaining accurate concentrations of free relaxin in the incubation wells, in spite of stringent methodology with limited handling. In either case, this is of general importance for *in vitro* experiments with relaxin.

The unchanged hydroxyproline concentrations found in the present study suggest that the collagen concentration was not affected dramatically. However, the hydroxyproline concentration does reflect the strength of the collagen, as collagen may still be present as fragments that would not demonstrate altered hydroxyproline concentrations. In addition, the decrease in strength after incubation with relaxin was not followed by histological changes in the organization of the collagen.

H1 relaxin m-RNA has been isolated from human fetal membranes (13), but the protein remains to be demonstrated in tissue. We found no significant effects of H1 relaxin on the fetal membranes. H1 relaxin has previously been reported as having 20% of the activity of H2 relaxin (16). An effect of such magnitude (20% of H2 relaxin) may, however, be undetected in the present study, as the mean values might suggest. To our knowledge a significant effect of H1 relaxin on human tissues remains to be demonstrated.

In conclusion, this study showed no statistically significant effect of H1 relaxin on the strength of the human fetal membranes, but H2 relaxin induced a biphasic weakening of the human fetal membranes over a limited concentration range. This decrease may be attributed to a relaxin-induced change in collagenases, because it can be inhibited by co-incubation with a synthetic collagenase inhibitor.

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