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Isolation, identification and biological activity of gastrin-releasing peptide 1–46 (oGRP$_{1-46}$), the primary GRP gene-derived peptide product of the pregnant ovine endometrium

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ABSTRACT

We have previously demonstrated that pregnant ovine endometrium expresses the gastrin-releasing peptide (GRP) gene at a high level following conceptus implantation. Here we report the isolation, characterization and biological activity of ovine GRP1–46, the primary product of this gene in the pregnant endometrium. Full thickness 125–140-day pregnant sheep uterus (term is 145 day) was homogenized in 80% acetonitrile/2% trifluoroacetic acid (1:7 ACN/TFA), concentrated on reverse-phase C18 cartridges and chromatographed successively on gel filtration (Sephadex G-50) and reverse-phase HPLC (C18 μBondapak). Purification was monitored by RIA. Purified GRP peptide was analysed by mass spectrometry giving a major mass ion at 4963 which corresponds exactly to GRP1–46. Other mass ions from pro-GRP did not contain a biologically active N-terminus or antigenic determinant. Proteolytic cleavage of pro-GRP to give rise to GRP$_{1-46}$ would require preferential cleavage at the Glu–Glu bond by a Glu-C2-like enzyme, rather than the trypsin-like and C-terminal amidation enzymes (PAM) that produce GRP$_{18-27}$ and GRP$_{1-27}$ in other tissues. GRP1–46 was synthesized and receptor binding and biological activity tested on a range of rodent and human cell lines that express GRP-related receptors GRPR, NMBR and BRS3. GRP1–46 bound GRPR and NMBR with low affinity, and mobilized inositol phosphate in cell lines expressing the GRPR and NMBR, but not BRS-3. This study describes a new processed product of the GRP gene, GRP1–46, which is highly expressed in the pregnant sheep endometrium and which acts as a weak agonist at the GRPR and NMBR.

1. Introduction

Gastrin-releasing peptides (GRPs) are the mammalian homologs of the frog skin peptide bombesin, and have a broad spectrum of regulatory functions in tissues as diverse as the central nervous system, pituitary and gastrointestinal tract [17]. The major recognized bioactive forms of GRP are the amidated GRP1–27 and 18–27. GRPs mediate these functions primarily via neurotransmission and also locally by paracrine or autocrine means. No hormonal role for this peptide family has yet been described, except for the presence of an immunoreactive GRP peptide product which circulates at high levels in the fetal and maternal circulation of the pregnant sheep [8].

We and others have previously shown that the pregnant ovine and bovine endometrium expresses the GRP gene, producing very large amounts of a translated and processed product which is different to the well characterized amidated bioactive peptides GRP1–27 and 18–27, as well as the C-terminally Gly extended forms [1,2,6,7,9,24,26]. Indeed this protein was by far the major stored and secreted form of pro-GRP processing in the pregnant sheep [26]. Using antisera directed against the amidated C-terminus of GRP1–27 (common to all mammalian species), we

Abbreviations: BSA, bovine serum albumin; C-terminus, carboxyl terminus; CNS, central nervous system; BRS-3, bombesin receptor subtype 3; GRPgastrin-releasing, peptide; GRPR, gastrin-releasing peptide receptor; HRP, horseradish peroxidase; SCLC, small cell lung cancer cells; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; NaN3, sodium azide; NMBR, neuromedin B receptor; RIA, radioimmunoassay; RP-HPLC, reverse-phase high pressure liquid chromatography; SacCel, silica beads with cellulose; TFA, trifluoroacetic acid.

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showed that the primary gene product synthesized by the ovine endometrium during pregnancy is a 5–6.5 kDa protein that cross-reacts weakly with our antiserum [26]. The peptide cannot be an N-terminally extended form of GRP, as the known processing products occur immediately C-terminal to the pro-GRP signal sequence, and cross-reactivity with other related gene products with homology to GRP such as NMB have been excluded [26]. This suggests that since an alternate GRP transcript has not been detected [27,28], the protein in question is most likely to be a C-terminally extended form of GRP, and that it is bound with low affinity by the detection antiserum. This observation, combined with difficulties in determining the molecular mass ion by mass spectroscopy has previously precluded unambiguous identification of this GRP product.

Recently we have tested a new GRP antiserum which was raised to the C-terminal region of GRP18–27 extended by glycine residue (GRP18–27gly). Unexpectedly, this antiserum bound avidly to the ovine pregnant endometrial GRP peptide, substantially reducing the detection threshold for monitoring purification by radioimmunoassay (RIA). Here we report the successful isolation, characterization and biological activity of the principal pro-GRP-derived processing product of the pregnant ovine endometrium, which corresponds to GRP1–46 (oGRP1–46).

2. Materials and methods

2.1. Isolation of oGRP1–46

2.1.1. Tissue extraction and initial purification

Late pregnant ovine endometrium was obtained from 125- to 140-day pregnant ewes after ethical culling. Protocols were assessed and passed by the appropriate institutional animal ethics committee. Three different extraction procedures were evaluated in terms of extraction efficacy of immunoreactive GRP eluting prior to the GRP1–27 standard at 5–6.5 kDa. The extraction conditions were 3% acetic acid, ice-cold acetonitrile/trifluoroacetic acid (TFA), and ice-cold formic acid/TFA. Ice-cold 80% acetonitrile/2% TFA extraction was found to be the most effective extraction medium.

Briefly, frozen endometrium was pulverized in liquid nitrogen using a mortar and pestle to produce a fine frozen powder. 2 g was immediately added to 10 vol. 80% acetonitrile/2% TFA and homogenized. After centrifugation (10,000 × *g* for 15 min) the supernatant was collected and the pellet re-extracted in the same solution. Supernatants were combined and acetonitrile evaporated under a continuous stream of air. The liquid phase was cooled on ice, then passed 3 times through a C18 Sep-pak reverse-phase cartridge which had been activated and washed according to the manufacturer's instructions (Millipore/Waters Rydalmere, Australia). GRP was eluted with 6 ml of 75% acetonitrile/0.05% TFA.

2.1.2. Gel filtration of semi-purified GRP

Sep-pak eluates were freeze dried and concentrated to <100 μl, reconstituted in 3% glacial acetic acid and chromatographed on Sephadex G-50 (1.5 cm × 90 cm) with 3% glacial acetic acid as the eluant. Fractions (100 × 1.4 ml) were collected, lyophilized and assayed for GRP immunoreactivity by RIA. The gel filtration column was calibrated with BSA (void volume), ovine GRP1–46, human GRP1–27 and human GRP18–27.

2.1.3. Reverse-phase high pressure liquid chromatography (RP-HPLC)

The GRP immunoreactive profile on gel filtration gave an overlapping immunoreactive doublet at fractions 40/41 and 44/45 (see Fig. 1A). This peak eluted between the void volume and human (h) GRP1–27. Each peak (2.8 ml) was freeze dried to <100 μl and separately chromatographed by RP-HPLC on a C18 μBondapak column (Millipore-Waters) eluted with a gradient of 0–70% acetonitrile/0.05% TFA. Peak fractions 40/41 and 44/45 each gave a single sharp Gaussian peak with identical elution position (~30% acetonitrile) containing 82 and 95 pmol immunoreactivity respectively (Fig. 1B). These were used for subsequent matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis.

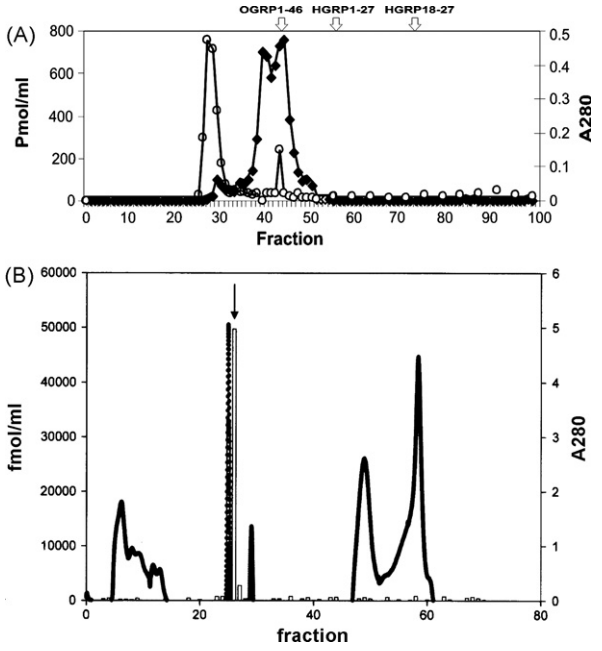

Fig. 1. (A) Gel filtration chromatography of protein extract of pregnant ovine endometrium. Open circles indicate the total protein elution profile (A280 nM) while filled rectangles show the immunoreactive GRP profile. The elution positions of human (h) GRP18–27 and hGRP1–27 as well as oGRP1–46 are indicated. (B) Reverse-phase C18 chromatography (μBondpak) of pooled fractions 40 and 41 from gel filtration step. The immunoreactive profile is indicated by the open bars with the peak fraction used subsequently for MALDI-TOF indicated with an arrow. Total protein is shown by the solid line.

2.1.4. Mass spectroscopy

The HPLC fraction was concentrated using a vacuum centrifugal concentrator and then 1 μl was mixed with 1 μl of matrix (α-cyano-4-hydroxy-cinnamic acid) and dried onto a sample plate followed by mass spectrometric analysis using the MALDI-TOF method on an Qstar mass spectrometer (Applied Biosystems, Foster City, CA).

2.2. RIA of GRP

Two RIAs were used to detect oGRP1–46; one used initially for primary extraction monitoring and directed to the amidated C-terminus of GRP18–27 and 1–27 (#R40), and the other used for final purification monitoring, and directed against gly-extended GRP18–27 (#8684; GRP18–27gly).

2.2.1. GRP #R40 RIA

The details of this assay have been previously published [4]. Briefly this antiserum detects amidated GRP of all mammalian species tested. ^{125}I-labeled Tyr^4-bombesin was used as the tracer and was prepared using iodogen (Pierce Chemical, Rockford, IL) followed by reduction with dithiothreitol to reverse methionine oxidation and purified by RP-HPLC. Assay cocktail was 100 μl tracer (3000–5000 cpm), 100 μl antibody (1:75,000 final dilution), sample and standard (2–2000 fmol/ml bombesin) made up to 1 ml with 0.02 M veronal buffer containing 0.1% BSA and 2 μM NaN_3. Bound from free counts were separated using either activated charcoal or SacCel (100 μl/tube). ID_{50} was 100 fmol/tube, intra-assay and inter-assay variation 4% and 14% respectively.