Human placental GnRH-like factors: parallel displacement in GnRH immuno- and receptor-binding assays can be caused by degradation of radiolabelled GnRH tracers

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Introduction

Gonadotrophin-releasing hormone (GnRH) acts in an autocrine/paracrine fashion as one factor in a complex regulatory system (Petraglia, 1997) which controls the secretion of a number of hormones by the human placenta (Merz et al., 1991; Siler-Khodr et al., 1991; Barnea et al., 1992). Radiolabelled GnRH analogues bind specifically, and with moderate affinity, to human placental membranes (Currie et al., 1981; Belisle et al., 1984; Iwashita et al., 1986; Bramley et al., 1992). The GnRH receptor gene is expressed in cytotrophoblast and syncytiotrophoblast cells (Lin et al., 1995; Wolfahrt et al., 1998), and appears to be similar to that expressed in the human pituitary (Kakar et al., 1995; Leung and Peng, 1996). Moreover, the gene for mGnRH is expressed in human placenta (Seeburg and Adelman, 1984; Seeburg et al., 1987; Radovick et al., 1990; Duello et al., 1993; Wolfahrt et al., 1998), and appears identical to the hypothalamic peptide (Osathanondh and Elkind-Hirsch, 1981; Tan and Rousseau, 1982; Seeburg et al., 1987; Zhuang et al., 1991), although GnRH-like peptides (Mathialagan and Rao, 1986a,b; Siler-Khodr, 1987; Zhuang et al., 1991) have also been isolated from human placental tissue.

More recently, post-translationally modified variants of GnRH have been described in placenta (Gautron et al., 1989; Currie et al., 1992), and other forms of GnRH have been shown to be present in mammals (mGnRH; Rissman et al., 1995; Kasten et al., 1996; Jimenez-Linan et al., 1997; Lescheid et al., 1997; Quanbeck et al., 1997), including the human (White et al., 1998), suggesting that other GnRH isoforms in addition to mGnRH may exist in extra-pituitary tissues, e.g. placenta. Moreover, we have shown previously that some [125I]-labelled GnRH isoforms (sGnRH = cGnRH II > mGnRH >> cGnRH I = lGnRH I) bound specifically to human placental membranes, but not to rat pituitary membranes (Bramley et al., 1992). Binding of salmon GnRH (sGnRH) and chicken GnRH (cGnRH) II compared favourably with that of the GnRH superagonists, buserelin and [D-Trp6] GnRH ethylamide ([D-Trp6] GnRH EtA) at all stages of gestation (Bramley et al., 1994).

GnRH radioreceptor or radioimmunoassays are sensitive to interference from peptides which degrade components of the assay (hormone tracer, antibody, receptor), giving the appearance of immuno- or receptor-active GnRH-like activity (Siler-Khodr et al., 1989). Hence, studies of extrapituitary GnRH often begin by extraction with denaturing agents (hot organic acid; extraction with alcohols or acetone) which give a good yield of the GnRH decapeptide, but almost certainly destroy the larger molecular weight forms of GnRH. Although human placental extracts do indeed inactive [125I]-labelled GnRH analogues (Menzies and Bramley, 1992; Bramley and Menzies, 1996), other studies in our laboratory have suggested the presence of a large, heat-labile GnRH-like activity in human
placental extracts which is unrelated to GnRH-degradation (T.A.Bramley and M.Mullen, unpublished data).

Since we wished to identify and purify all form(s) of GnRH and GnRH-like factors secreted by the human placenta, we first required a robust assay which would enable the measurement of various GnRH forms by radioimmunoassays and/or radioreceptor assays in fractions from un-denatured human placental extracts. In the course of these studies, the impact of inactivation of the radiolabelled GnRH tracers utilized became increasingly obvious; we now report the effects of un-denatured extracts of human term placenta on GnRH tracer integrity, and describe interference by placental peptidase(s) on GnRH measurements by immuno- and receptor-binding assays.

Materials and methods

Materials

Sephadex G25 (fine) was obtained from Pharmacia Ltd (Milton Keynes, Bucks, UK), and from Sigma Chemical Co Ltd (Poole, Dorset, UK). QAE-sepharose and CM-sepharose were obtained from Pharmacia. Thin layer chromatography (TLC) plates (polyethyleneimine cellulose on polyester backing, with or without fluorescent indicator) were obtained from Sigma. All other fine chemicals, enzyme inhibitors and reagents were from either Sigma or BDH (Poole, Dorset, UK). Radiolabelled sodium iodide (Na$^{125}$I) was obtained from Amersham International plc (Little Chalfont, Bucks, UK).

Lamprey GnRH I (lg GnRH I) and salmon GnRH (sGnRH) were the generous gifts of Dr J.King (MRC Regulatory Peptides Research Unit, University of Cape Town, South Africa). cGnRH II was purchased from Peninsula Laboratories, Belmont, CA, USA. The GnRH agonist buserelin (l-D-Ser (tBu)$^3$) 1–9 GnRH ethylamide) was the kind gift of Dr J.Sandow (Hoescht AG, Frankfurt, Germany). All other GnRH analogues were purchased from Sigma. A conformation-dependent anti-GnRH antibody (R 1245, which recognizes all isoforms of GnRH except lg GnRH I) was the kind gift of Dr G.D.Niswender (Fort Collins, Colorado, USA; see Bramley et al., 1992) and a second GnRH-specific antibody (HU 60) was the generous gift of Dr Henry Urbanski (Oregon Regional Primate Center, Portland, OR, USA). The specific anti-cGnRH II antibody (Ab 10.2; Sharp et al., 1987) was a gift of Professor P.Sharp (Roslin Institute, Scotland, UK).

Preparation and measurement of specific activities of $^{125}$I-labelled GnRH tracers

Peptides were radioiodinated using a glucose oxidase/lactoperoxidase method, and were purified by chromatography on Sephadex G25 columns (Bramley et al., 1992). Specific activities of radiolabelled mono-iodinated GnRH isomorph preparations were estimated by a self-displacement assay (Clayton, 1983) using the conformation-dependent anti-GnRH antibody (R 1245; 85–1100 Ci/g; $n = 9$ separate GnRH isoform preparations). Specific activities of GnRH agonist tracers were measured by self-displacement assay of binding to homogenates of immature female rat pituitary glands (350–1105 Ci/g, $n = 12$ preparations).

Tissues

Pituitary

Rat pituitaries were obtained from immature (28–35 day) female Sprague–Dawley rats killed by CO$_2$ asphyxiation. Pituitaries were excised and homogenized in ice-cold 0.3 mol/l sucrose–10 mmol/l Tris–1 mmol/l EDTA, pH 7.4 (SET medium; two glands/ml) using a loose-fitting all-glass Dounce homogenizer. Protease inhibitors were omitted from preparative buffers, as these may affect the placental GnRH/GnRH receptor system (Bramley et al., 1999). Aliquots (2 ml) of homogenate were snap-frozen in solid CO$_2$, and stored at $-20^\circ$C until required.

Placentae

Human placentae were obtained from normal women following spontaneous vaginal delivery ($n = 12$) or elective Caesarian section at term ($n = 1$). Placentae from early gestation pregnancies (8 weeks) were terminated using Mifepristone (RU 486) followed by induction of labour using Gemeprost pessaries (Norman et al., 1992). Pregnancies of 15–16 weeks gestation were generally terminated using prostaglandin alone. Approval for these studies was obtained from the Human Reproduction Ethical Committee of the Lothian Health Board Hospitals Trust. Placental villous tissue was dissected, washed extensively in ice-cold isotonic phosphate-buffered saline (PBS; Flow Laboratories, Irvine, Scotland, UK) to reduce blood contamination, and villi were minced and homogenized in ice-cold SET buffer (5 ml/g) using a Polytron homogenizer (two 10 s bursts at full speed, separated by a 1 min cooling period in ice). After filtration through four layers of cheesecloth, homogenates were centrifuged at 1000 g for 10 min ($4^\circ$C) to remove nuclei and cell debris. Supernatants were re-centrifuged at 100 000 g for 60 min in a Sorvall OTD-50 refrigerated ultracentrifuge ($4^\circ$C). The microsomal pellets obtained were gently rehomogenized (5–10 strokes in a loose Dounce homogenizer) in SET medium, and supernatants (cytosol) and membranes were stored in 2 ml aliquots at $-70^\circ$C or in liquid nitrogen.

Methods

Protein was measured by a previously described method (Lowry et al., 1951), using crystalline bovine serum albumin (BSA) as a standard.

Measurement of Specific Binding of $^{125}$I-labelled GnRH

Binding of radiolabelled GnRH agonists and isoforms to human placental membranes was measured by incubation of triplicate aliquots (0.5–1.0 mg protein/tube) at $20^\circ$C for 1 h in a 0.5 ml incubation system containing 40 mmol/l Tris–HCl, pH 7.4, 0.5% BSA and 100 000 cpm of the appropriate $^{125}$I-labelled GnRH tracer. Non-specific binding was measured in duplicate in the presence of 10 µg of unlabelled buserelin. Bound hormone was recovered by immunoglobulin G (IgG)–polyethylene glycol (PEG) precipitation (Bramley et al., 1985), and pellets counted for $^{125}$I in a Packard Cobra™ II γ-counter (Meriden, CT, USA) at an efficiency of 75%. The difference between binding in the presence and absence of unlabelled GnRH agonist represented specific binding (normally adjusted to 15–35% of total counts added). Controls without tissue, with and without unlabelled GnRH agonist, were included to correct for displacement of tracer from assay tubes by cold analogue (usually 0.5–1% of total counts added).

Specific binding of $^{125}$I-labelled GnRH agonists to rat pituitary homogenates or membranes was measured as described above, except that incubation was for 4 h at $4^\circ$C.

Measurement of GnRH tracer inactivation

Aliquots of placental cytosol (5–100 µl) were incubated in a 0.5 ml system containing 0.1% (w/v) BSA, 40 mmol/l Tris–HCl buffer, pH 7.4, and 30–100 000 cpm of $^{125}$I-labelled GnRH isoform or analogue, as indicated. After incubation, GnRH degradation was assessed by a variety of methods.

High-resolution liquid chromatography (HRLC)

Aliquots (200 µl) of incubation media or dextran-charcoal supernatant fractions (see below) were applied through a 1 ml injection loop
to a BioRad (Hemel Hempstead, UK) HRLC 500 series liquid chromatograph system (RoSiL C18 HL 5 µm column; 0.46 × 25 cm) and eluted isocratically (1.5 ml/min) for 10 min with 5% acetoni trile in TEAF (0.25 mol/l formic acid adjusted to pH 6.5 with triethanolamine; Sherwood et al., 1991). Acetonitrile was then increased to 60% over 55 min (1% change per min). Fractions (1 ml) were collected and 125I counted in a Packard Cobra™ II γ-counter at an efficiency of 75%.

**Thin layer chromatography (TLC)**

Aliquots (5 µl) of incubation media or dextran-coated charcoal (DCC)-supernatants were spotted onto polyethylenimine cellulose TLC plates, developed in 0.1 mol/l ammonium bicarbonate (pH 7.8), dried, wrapped in cling-film, and placed in a phosphorimagery cassette. After exposure for 2–5 days, plates were scanned in a Molecular Dynamics (Sunnyvale, CA, USA) phosphorimagery. Spot intensities and the proportion of radioactivity present in different regions of the TLC plate were calculated using ImageQuant Software (Molecular Dynamics; IQMac, version 1.2).

**Antibody binding**

Binding of [125I]-labelled GnRH tracers (20–30 000 cpm per tube) to a conformational anti-GnRH antibody (R 1245; final dilution, 1:30 000), anti-GnRH antiserum (HU 60; final dilution, 1:20 000–1:50 000) or specific anti-cGnRH II antibody (1:30 000) was performed as described previously for EL14 anti-GnRH antiserum (Bramley et al., 1992).

**Receptor binding**

Specific binding of GnRH isoform or agonist tracers to human placental microsomes or rat pituitary receptors was measured as superagonist tracers to term placental microsomes. Specific aliquots of human term placental microsomes were incubated with [125I]-labelled mammalian GnRH (mGnRH; ○), chicken GnRH II (cGnRH II; ●), salmon GnRH (sGnRH; △), [D-Trp6] GnRH EtA (▲) or buserelin (■) for 2 h at 20°C, in the absence, or in the presence of increasing concentrations of a human term placental cytosol fraction. Points shown are mean ± SEM for a single representative experiment in triplicate.

**Figure 1.** Effects of inclusion of placental cytosol on specific binding of [125I]-labelled gonadotrophin-releasing hormone (GnRH) isoforms and agonists to human placental microsomes. Triplicate aliquots of human term placental microsomes were incubated with [125I]-labelled mammalian GnRH (mGnRH; ○), chicken GnRH II (cGnRH II; ●), and salmon GnRH (sGnRH; △). Specific binding of GnRH isoforms or agonists by 50% (IC50) were calculated from plots of specific binding versus log10 (placental extract added). The values were expressed as mean ± SEM for a single representative experiment in triplicate.

**Calculations and statistics**

Concentrations of placental extract required to reduce specific binding of GnRH isoforms or agonists by 50% (IC50) were calculated from plots of specific binding versus log10 (placental extract added). Statistical significance of differences between means was estimated by Student’s t-test with Bessel’s correction for small numbers, or by Wilcoxon’s rank order test. *P < 0.05* was considered to be statistically significant.

**Results**

**Effects of human placental extracts on binding of GnRH tracers**

There was a marked difference in the effects of placental cytosol on the binding of [125I]-labelled GnRH isoforms and GnRH superagonist tracers to term placental microsomes. Specific binding of [125I]-labelled mGnRH, sGnRH and cGnRH II decreased in a concentration-dependent manner with increasing placental cytosol (Figure 1). However, even high concentrations of the same cytosol preparation had little or no effect on the binding of radiolabelled buserelin or [D-Trp6] GnRH EtA (Figure 1). Further detailed studies showed that term human placental cytosol preparations decreased binding of radioiodinated mGnRH to placental microsomes in a dose-dependent manner (Figure 2C), and competed for [125I]-labelled cGnRH II (Figure 2A) and sGnRH binding sites (Figure 2B) in parallel with displacement curves for unlabelled GnRH isoform/agonist. However, the same placental extract had little or no effect on the binding of radiolabelled GnRH agonists (buserelin; Figure 2D, and [D-Trp6] GnRH EtA; Figure 2E). Despite the lack of effect of placental cytosol fractions on [125I]-GnRH agonist binding to human placental microsomes, placental cytosol preparations did compete in parallel with GnRH agonist standard for the binding of radiolabelled GnRH agonist (buserelin or D-Trp6 GnRH EtA) to rat pituitary membranes (Figure 2F, Table I), though at higher concentrations (IC50; 3–20 mg protein; *n = 6*) than those required to inhibit cGnRH II binding to placental receptors (IC50; 0.1–0.3 mg protein; *n = 8*).

**Do GnRH isoforms and agonists bind differently to placental receptors?**

Differential binding of GnRH agonists and isoforms may reflect a species difference (rat versus human GnRH receptors), or differences between pituitary and extra-pituitary GnRH-receptors. Alternatively, agonist and isoform ligands may bind differentially to distinct GnRH-receptor subtypes, to post-translationally modified forms of the GnRH-receptor, or to different receptor states of the same placental GnRH-receptor(s). The putative human placental GnRH-like factor
Figure 2. Parallel inhibition of specific binding of $^{125}$I-labelled gonadotrophin-releasing hormone (GnRH) ligands to human placental microsomes by placental cytosol and unlabelled GnRH isoforms and agonists. Triplicate aliquots of human term placental microsomes were incubated with $^{125}$I-labelled GnRH binding ligands for 2 h at 20°C, in the absence, or in the presence of increasing concentrations of a human term placental cytosol fraction or unlabelled GnRH analogues. Specific binding data are plotted as percentages relative to controls with no cytosol or GnRH peptides. (A) Binding of chicken GnRH II (cGnRH II) tracer to placental microsomes with placental cytosol (●) or unlabelled buserelin (○) or cGnRH II (△); (B) Binding of salmon GnRH (sGnRH) tracer to placental microsomes with placental cytosol (●) or unlabelled buserelin (○) or sGnRH (△); (C) Binding of mGnRH tracer to placental microsomes with placental cytosol (●) or unlabelled mammalian GnRH (mGnRH; ○); (D) Binding of buserelin tracer to placental microsomes with placental cytosol (●) or unlabelled buserelin (○); (E) Binding of [D-Trp$^6$] GnRH ethylamide (EtA) tracer to placental microsomes with placental cytosol (●) or unlabelled [D-Trp$^6$] GnRH EtA (○). Points shown are means ± SEM for a single representative experiment in triplicate. (F) Binding of radiolabelled [D-Trp$^6$] GnRH EtA (△, ▲) or buserelin (○, ●) tracer to rat pituitary receptors with placental cytosol (△, ○) or unlabelled GnRH agonist (solid symbols). Points are means of 2–4 experiments in triplicate. Standard error bars are omitted for clarity, but were always less than ±10%.

(hpGnRH) in cytosol fractions may then compete preferentially for a GnRH isoform-specific state of the receptor, rather than to a GnRH agonist-binding state. We therefore extended our studies to compare the specificity of the placental GnRH-receptor for a range of GnRH isoforms, agonists, antagonists and other peptides (with or without gonadotrophin-releasing activity), using both radiolabelled GnRH isoforms and agonists as binding ligands.
GnRH isoforms and superactive agonists had similar binding potencies to placental membranes with either [125I]-labelled GnRH agonist (Figure 3A,B) or GnRH isoform tracers (Figure 3C–E) as binding ligands. The binding affinities of the rat pituitary and human placental GnRH receptors were similar for the GnRH isoforms tested, but differed markedly for GnRH agonists and antagonists (Figures 3A,D,E; Table II). Similar data were obtained with [125I]-labelled sGnRH (Figure 3E) and [D-Trp6] GnRH EtA (data not shown) as the binding ligands.

In contrast, a peptide isolated from human follicular fluid with GnRH-like activity but which was unrelated structurally to GnRH (Li et al., 1987) failed to compete for buserelin (Figure 3B) or cGnRH II binding (Figure 3C) to placental membranes. Moreover, a number of peptides with an N-terminal pGlu (gastrin I, hydra peptide, neurotensin), a C-terminal Gly-amide (thymosin a1) or with both an N-terminal pGlu1 and a C-terminal amide residue (thyrotrophin-releasing factor, bombesin, gastrin II, physalaemin, serum thymic factor, adipokine hormone II, caerulein, edeoisin, gastrin releasing peptide, pancreastatin) did not compete for placental GnRH binding sites (Figures 3B,C and unpublished data). No inhibition was seen with other unrelated peptides (epidermal growth factor, oxytocin, insulin, angiotensin I, II and III; data not shown). These experiments indicated similar binding affinities and specificities of placental membrane receptors with either radiolabelled GnRH agonists and GnRH isoforms.

Term placental cytosol fractions also gave parallel displacement to GnRH isoforms in a GnRH-specific radioimmunoassays (Figure 4A). Concentrations of cytosol required to inhibit specific binding of [125I]-labelled cGnRH II to placental receptors or GnRH antibody by 50% (IC50) were similar (Table I). Moreover, placental cytosols from earlier stages of gestation (8, 15 and 16 weeks gestation) also showed high immunoreactivity (Figure 4B). However, isolation of 15-week placental cytosol fraction in the presence of a cocktail of protease inhibitors markedly reduced GnRH-like immunoreactivity (Figure 4B).

**Effects of cytosol on integrity of [125I]-labelled GnRH tracers**

The latter observation led us to test the inhibition of binding of [125I]-labelled cGnRH II or buserelin to human term placental membranes by a number of different term placental cytosols, prepared both in the absence (protocol 1) and in the presence of a cocktail of peptidase inhibitors (protocol 2). There was a significant increase in the concentration required to inhibit buserelin tracer binding when cytosol was prepared with protease inhibitors (IC50, 0.73 ± 0.14 mg protein; n = 11) compared with preparations without inhibitors (IC50, 0.15 ± 0.05 mg protein; n = 6; P < 0.05). A similar reduction was observed for inhibition of [125I]-labelled cGnRH II binding (IC50, 0.18 ± 0.06 versus 0.07 ± 0.01 mg protein respectively; n = 6), though this failed to reach statistical significance.

Since GnRH superagonists are designed to have increased resistance to proteolytic degradation (D-aminoacid substitution at Gly6; C-terminal ethylamide), an alternative explanation for the differences in potency of placental extracts towards binding of radiolabelled GnRH isoforms and agonists (as well as the reduced immuno- and receptor-binding activity of placental cytosol prepared with and without protease inhibitors) is that placental cytosol fractions possess enzyme(s) capable of inactivating GnRH isoforms, but not GnRH agonists. We therefore compared the effects of incubation of radiolabelled GnRH isoforms and agonists with placental extracts using a variety of different techniques to estimate loss of binding ligand integrity.

**High resolution liquid chromatography**

HRLC demonstrated a marked change in tracer integrity following incubation with cytosol, with the appearance of a major product having a much shorter retention time than intact cGnRH II tracer (Figure 5A). Incubation of placental extracts with [125I]-labelled [D-Trp6] GnRH EtA also demonstrated the appearance of a novel peak (Figure 5B). However, the amount of novel product formed was much lower than with [125I]-labelled cGnRH II, and more intact tracer remained, suggesting lower degradation of the agonist compared with the GnRH isoform. Treatment of tracer incubated in the absence of cytosol with DCC adsorbed almost all the intact cGnRH II and [D-Trp6] GnRH EtA tracer remaining after incubation: however, the magnitude of the novel tracer peaks generated during incubation with cytosol was decreased only slightly by DCC adsorption (Figures 5A,B).
Figure 3. Comparison of the specificities of gonadotrophin-releasing hormone (GnRH) isoform and agonist tracers for human placental microsomal GnRH-binding sites. Triplicate aliquots of human term placental microsomes were incubated with \(^{[125]}\text{I}\)-labelled GnRH binding ligands for 2 h at 20°C, in the absence, or in the presence of increasing concentrations of unlabelled GnRH analogues or peptides. Antagonist structures were as shown in the legend to Table II. (A) Radiolabelled buserelin tracer with unlabelled buserelin (○), mammalian GnRH (mGnRH; ○), chicken GnRH I (cGnRH I; △), cGnRH II (▲), [D-Trp\(^6\)] GnRH ethylamide (EtA; ●), Antagonist 1 (◇), Antagonist 2 (■) and Antagonist 3 (□). (B) Radiolabelled buserelin tracer with unlabelled buserelin (○), [D-Trp\(^6\)] GnRH EtA (◊), Lı’s peptide (Δ) and a composite of a number of non-GnRH peptides (TRF, gastrin I, gastrin II, hydra peptide, neurotensin, thymosin α₁, bombesin, physalaemin, serum thymic factor, adipokinetic hormone II, caerulein, eldeoisin, gastrin releasing peptide and pancreastatin; ▲). Points are means ± SEM for 2–8 separate experiments in triplicate. (C) Radiolabelled cGnRH II tracer with unlabelled cGnRH II (○), cGnRH I (◇), mGnRH (△), sGnRH (▲), dogfish GnRH (□), l GnRH 1 (■), catfish GnRH (●) and a number of non-GnRH peptides (see B above; ◇). (D) Radiolabelled cGnRH II tracer with unlabelled cGnRH II (○), buserelin (○), [D-Ala\(^6\)] GnRH EtA (△), [D-Trp\(^6\)] GnRH EtA (▲), Antagonist 1 (■), Antagonist 2 (□), Antagonist 3 (◇), Antagonist 4 (●) and Antagonist 5 (X). (E) Radiolabelled sGnRH tracer with unlabelled sGnRH (●), cGnRH I (◇), cGnRH II (△), buserelin (▲), [D-Trp\(^6\)] GnRH EtA (□), Antagonist 1 (■) and Antagonist 5 (●). Points are means for 2–4 separate experiments in triplicate.

Thin layer chromatography
Since HRLC was time-consuming, expensive and could handle only a few samples per day, the integrity of cGnRH II tracers was studied further by TLC on polyethyleneimine cellulose TLC plates. Degradation of cGnRH II and [D-Trp\(^6\)] GnRH EtA tracers was markedly reduced by incubation at lower
temperature (0°C) or with boiled cytosol (Table III). Intact cGnRH II tracer, and tracer incubated in the absence of cytosol at 20°C, remained at the application origin (Figure 6A; lane 1). The spot at the origin (intact tracer) was markedly reduced when controls incubated in the absence of cytosol were adsorbed by DCC (lane 2). Following incubation with placental cytosol, radioactivity remaining at the origin was reduced, and a novel major spot appeared (Rf, 0.45; lane 3). However, after incubation with cytosol followed by DCC adsorption, the intensity of the novel Rf 0.45 spot was essentially unchanged (lane 4), whereas activity at the origin (intact tracer) was abolished. Boiling of cytosol prior to incubation with cGnRH II tracer prevented the appearance of the Rf 0.45 spot, and radioactivity was recovered once more at the origin (lane 5; Table III). DCC adsorption after incubation with boiled cytosol (lane 6) reduced radioactivity substantially.

[125I]-labelled [D-Trp6] GnRH EtA also remained at the origin following TLC (Figure 6B; lane 1), and was adsorbed by DCC treatment (lane 2). An additional spot appeared after incubation with placental cytosol (Figure 6B; lane 3), but the low Rf of the product formed (Rf < 0.05) made it difficult to resolve from intact tracer remaining at the origin. However, DCC adsorption of tracer incubated with placental extract decreased the intensity of the spot at the origin (lane 4), allowing the product to be seen clearly. Tracer was recovered at the origin after incubation with boiled cytosol (Figure 6B; lane 5), and was adsorbed by DCC (lane 6). Similar changes were seen with radioiodinated buserelin tracers (data not shown).

**Adsorption of [125I]-labelled GnRH tracers**

The data presented in Figures 5 and 6 suggested that differential adsorption of intact and inactivated tracers to dextran or DCC may provide a rapid screen for the measurement of GnRH tracer degradation. We therefore investigated adsorption of intact and degraded GnRH tracers to different membranes and chromatography gels. Ion exchange chromatography (DEAE- and CM-sepharses) of radiolabelled GnRH agonist and isoforms following incubation with placental cytosol resolved intact and inactivated tracers, but resolution of degraded and un-degraded peptides was variable and dependent on the ionic strength of the elution buffer (data not shown).

[125I]-labelled cGnRH II incubated without placental cytosol adsorbed strongly to Sephadex G25, eluting in a broad peak which was retarded beyond the total volume (Ve) of the column (Figure 7A,B). Fractions from the tracer peak bound well to
both a specific anti-cGnRH II antibody (Figure 7A) and a conformation-specific anti-GnRH antibody (Figure 7B), demonstrating that this peak was intact cGnRH II tracer. After incubation with placental cytosol, however, the main peak of radioactivity was no longer retarded, but eluted in the total volume of the column (Figure 7C,D). This material failed to bind to either anti-GnRH antiserum (Figure 7C,D), suggesting it consisted of degraded products of $[^{125}\text{I}]$-labelled GnRH. cGnRH II tracer incubated with boiled cytosol gave identical elution profiles to tracer incubated without cytosol, and bound well to both anti-GnRH antisera; data not shown.

Adsorption of cGnRH II and mGnRH tracer to Whatman filter paper, cellulose nitrate and nylon membranes was significantly reduced by incubation with placental cytosol, but not by boiled cytosol (Table IV). Incubation with cytosol had a much smaller effect on the adsorption of $^{125}\text{I}$-[D-Trp$^6$] GnRH EtA.

**Correlation of DCC adsorption with binding to anti-GnRH antibody and GnRH receptor**

We have shown above that DCC adsorbed intact, but not degraded GnRH tracers (Figures 6 and 7). We therefore compared the effects of cytosol on degradation of cGnRH II tracer measured by DCC adsorption with the effects of cytosol in a human placental GnRH radioreceptor assay and a cGnRH II-specific radioimmunoassay. $[^{125}\text{I}]$-labelled cGnRH II not adsorbed by DCC increased in a dose-related manner with increasing cytosol concentration (Figure 8A). Placental cytosol also reduced $[^{125}\text{I}]$-labelled cGnRH II binding to term placental.

### Table III. Recovery of intact $[^{125}\text{I}]$-labelled chicken gonadotrophin-releasing hormone (GnRH) II and [D-Trp$^6$] GnRH ethylamide (EtA) and the products formed following incubation with or without term placental cytosol. Data are given as mean ± SEM for 3–5 separate experiments with different tracer preparations and term cytosols

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nd = not determined.

*Significantly different from control ($P < 0.001$).
membranes (Figure 8B) and to anti-GnRH antibody (HU 60) in a dose-dependent manner (Figure 8C). The lack of specific binding of \([^{125}\text{I}]\)-labelled cGnRH II (or [D-Trp\(^6\)] GnRH EtA tracer; data not shown) after incubation with cytosol alone followed by polyethylene glycol precipitation (Figure 8D) indicated that displacement of tracer binding in the radio-immunoassays and radioreceptor assays was not due GnRH-receptor on unsedimented placental membrane vesicles in cytosol fractions. There was a good negative correlation between radioactivity recovered in the supernatant after DCC adsorption, and binding to both the placental membrane GnRH-receptor (Figure 8E; \(r^2 = -0.97\), \(P < 0.001\)) and to anti-GnRH antibody (Figure 8F; \(r^2 = -0.98\), \(P < 0.001\)).

**Discussion**

Human term placental cytosol fractions decreased the specific binding of \([^{125}\text{I}]\)-labelled GnRH isoforms to human placental microsomes in a dose-dependent fashion (Figures 1 and 2A–C; Table I). However, placental extracts failed to inhibit the binding of radiolabelled GnRH agonists to placental GnRH-receptors (Figures 1 and 2D,E; Table I, although cytosol did inhibit \([^{125}\text{I}]\)-labelled GnRH agonist binding to rat pituitary membranes at higher concentrations; Figure 2F and Table I). One possible explanation for the different effects of placental extracts on GnRH isoform and agonist binding to placental membranes is that isoform and agonist tracers bind differently to placental receptors (Tensen et al., 1997), or to different

![Figure 7](image-url). Integrity of \([^{125}\text{I}]\)-labelled chicken gonadotrophin-releasing hormone (cGnRH) II after incubation in the (A, B) absence or (C, D) presence of human placental cytosol assessed by Sephadex-G25 chromatography. Radiolabelled cGnRH II was incubated in (A, B) the absence or (C, D) presence of aliquots of a human term placental cytosol preparation. Aliquots were then subjected to chromatography on precalibrated Sephadex-G25 columns, and counted for \(^{125}\text{I}\) (○). Triplicate aliquots (100 µl) of each fraction were incubated for 8 h in Tris–BSA with 1:50 000 diluted anti-cGnRH II antibody (A, C) or a conformational GnRH antibody (R 1245; B, D). Bound hormone was separated by IgG/PEG, and selects counter for antibody-bound \(^{125}\text{I}\)-cGnRH II (●). \(V_0\) = void volume (Blue Dextran); \(V_t\) = total volume of column (Na\(^{125}\text{I}\)).

**Table IV.** Adsorption of \([^{125}\text{I}]\)-labelled chicken gonadotrophin-releasing hormone (cGnRH) II, mammalian GnRH (mGnRH) and [D-Trp\(^6\)] GnRH ethylamide (EtA) tracers to different supports following incubation with boiled or unboiled term placental cytosol. Figures are given as mean ± SEM of percentage binding relative to controls incubated without cytosol (data from 2–3 separate experiments in triplicate).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage tracer bound (relative intensity of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cGnRH II</td>
</tr>
<tr>
<td>Placental cytosol</td>
<td></td>
</tr>
<tr>
<td>unboiled</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>boiled</td>
<td>109 ± 4</td>
</tr>
<tr>
<td>Hibond membrane</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Whatman paper</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>97 ± 6</td>
</tr>
<tr>
<td></td>
<td>9 ± 6</td>
</tr>
</tbody>
</table>
receptor subtypes (Troskie et al., 1998), and that hpGnRH is more specific for the isoform-binding site than for the agonist-binding site. Despite human placental GnRH-receptor mRNA being very similar to that expressed in the pituitary gland (Kakar et al., 1995; Leung and Peng, 1997), there are certainly differences in binding specificity (Table II) and affinity (Currie et al., 1981; Belisle et al., 1984; Iwashita et al., 1986; Bramley et al., 1992, 1994) between rat pituitary and human placental GnRH-receptors. Moreover, other forms of GnRH are active in extra-pituitary human tissues (Gautron et al., 1989; Pati and Habibi, 1995). However, the specificities and affinities of rat and human pituitary GnRH receptors are similar (Wormald et al., 1985), suggesting ligand specificity differences reflect properties of pituitary versus extra-pituitary GnRH receptors rather than species differences. Receptors with distinct binding properties and/or ligand specificities may arise through expression of different GnRH receptor subtypes (Troskie et al., 1998) or differential splicing of the gene, but to date, distinct forms of GnRH-receptor have not been reported in human placenta.

We failed to detect any significant differences of the human placental receptor in binding ligand affinity or specificity for a range of peptides, using both [125I]-labelled GnRH isoforms or agonists as binding ligands. The different GnRH isoforms, agonists and antagonists tested had similar specificity profiles for all radiolabelled tracers used to measure binding to placental membranes (Figure 3). Interestingly, peptides with pGlu1 and/
or C-terminal amides failed to affect binding of either GnRH agonist (Figure 3B) or GnRH isoform (Figure 3D). Such peptides should compete for the active sites of pyroglutamyl peptidase and carboxypeptidase respectively. Hence, our data suggest that GnRH degradation under these conditions does not involve these enzymes.

G-proteins are present in the human placenta, and guanine nucleotides can modulate the affinity of extra-pituitary GnRH receptors (Segal-Abramson et al., 1992; Imai et al., 1996). Thus, differences in G-protein state, or coupling of receptors to different G-proteins (Delahaye et al., 1997), may account for some of the differences observed between GnRH isoform and agonist binding. However, GTP, GDP, GMP, GTPγS, GDPβS, or cGMP at concentrations up to 5 mmol/l had no effect on displacement curves of [125I]-labelled agonist or cGnRH II binding to placental membranes by unlabelled GnRH or GnRH agonists (data not shown), suggesting that G-protein coupling was not responsible for placental GnRH-receptor specificity differences.

Although GnRH-like peptides have been isolated from extra-pituitary tissues, and a GnRH-like factor was isolated from ovarian extracts of several species (including human; Aten et al., 1987), this was subsequently shown to be a histone protein (Aten and Behrman, 1989). However, a number of different histones (at concentrations up to 10 μg per tube) failed to inhibit binding of [125I]-labelled buserelin or cGnRH II to human placental or rat pituitary membranes (data not shown). Moreover, GnRH-binding to placental membranes was not inhibited by a human ovarian follicular fluid peptide structurally unrelated to GnRH (Li et al., 1987), emphasizing the specificity of placental receptors for molecules with a GnRH-like structure.

Isolation of placental cytosol in the presence of a cocktail of peptidase inhibitors abolished the apparent GnRH-like immunoactivity of a 15-week placental cytosol (Figure 4B) and reduced the ability of term placental cytosol preparations to inhibit GnRH agonist and isoform binding to term placental membranes, indicative of an effect of protease degradation during the assay. Indeed, a high molecular weight human placental GnRH-like factor was shown to be a protease (C-ase 1; Siler Khodr et al., 1989) which can degrade GnRH and other peptides.

Inactivation of [125I]-labelled GnRH isofoms tracers by human placental extracts was observed using a battery of measures of degradation, including HRLC (Figure 5), TLC (Figure 6), gel permeation chromatography (Figure 7), and adsorption by DCC (Figures 5, 6 and 8; Table III) and other supports (Table IV), and by ion exchange chromatography (unpublished data). We found an excellent negative correlation (P < 0.001) between tracer degradation (assessed by adsorption to DCC) and inhibition of GnRH binding to GnRH-antibody (Figure 8F) or placental receptor (Figure 8E). However, GnRH agonist tracers always appeared to be more resistant to degradation than GnRH isoform tracers (Figures 5 and 6; Tables III and IV).

In conclusion, we have demonstrated a strong negative correlation between the degradation of an [125I]-labelled GnRH isoform tracer by human placental extracts and apparent immuno- and receptor-binding GnRH-like activity (Figures 8E,F). However, whilst resistance of agonist tracers to peptidase degradation may account for the lack of effect of placental extract on the binding of agonists to placental membranes, these extracts did suppress binding of two GnRH agonist tracers to rat pituitary membranes (Figure 2F), suggesting that placental cytosol effects were not wholly attributable to tracer degradation. Indeed, the properties of C-ase 1 (Siler Khodr et al., 1989) differ significantly from our data on human placental cytosolic GnRH-degrading activities, and although inclusion of peptidase inhibitors significantly reduces the effects of placental extracts on both radioreceptor and radio-immunoassays, some hpGnRH-like activity persists under conditions of minimal GnRH tracer degradation (T.A.Bramley and G.S.Menzies, unpublished data). Such GnRH-like activity may be due to GnRH-binding protein(s) such as those in previous reports (Flanagan et al., 1996; Siler-Khodr et al., 1997). The isolation of GnRH-like factors in undenatured placental extracts awaits the development and validation of robust GnRH radioimmunoassays and radioreceptor assays which are insensitive to peptidase interference. Studies of the effects of different protease inhibitors on the degradation of GnRH binding ligands (GnRH isofoms and agonists) may facilitate the development of such assays.

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