Insulin-like growth factor (IGF)-I binding to a cell membrane associated IGF binding protein-3-acid-labile subunit complex in human anterior pituitary gland

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Abstract

The binding characteristics of $[^{125}\text{I}]$insulin-like growth factor (IGF)-I were studied in human brain and pituitary gland. Competition binding studies with DES(1-3)IGF-I and R$^3$-IGF-I, which display high affinity for the IGF-I receptor and low affinity for IGF binding proteins (IGFBPs), were performed to distinguish $[^{125}\text{I}]$IGF-I binding to IGF-I receptors and IGFBPs. Specific $[^{125}\text{I}]$IGF-I binding in brain regions and the posterior pituitary was completely displaced by DES(1-3)IGF-I and R$^3$-IGF-I, indicating binding to IGF-I receptors. In contrast, $[^{125}\text{I}]$IGF-I binding in the anterior pituitary was not displaced by DES(1-3)IGF-I and R$^3$-IGF-I, suggesting binding to an IGF-binding site that is different from the IGF-I receptor. Binding affinity of IGF-I to this site was about 10 fold lower than for the IGF-I receptor. Using western-immunoblotting we were also unable to detect IGF-I receptors in human anterior pituitary. Instead, western-immunoblotting and immunoprecipitation experiments showed a 150-kDa IGFBP-3-acid labile subunit (ALS) complex in the anterior pituitary and not in the posterior pituitary and other brain regions. RT-PCR experiments showed the expression of ALS mRNA in human anterior pituitary indicating that the anterior pituitary synthesis ALS. In the brain regions and posterior pituitary IGFBP-3 was easily washed away during preincubation procedures as used in the $[^{125}\text{I}]$IGF-I binding experiments. In contrast, the IGFBP-3 complex in the anterior pituitary could not removed by these washing procedures. Our results indicate that the human anterior pituitary contains a not previously described tightly cell membrane-bound 150-kDa IGFBP-3-ALS complex that is absent in brain and posterior pituitary.
Introduction

The Insulin-like growth factor (IGF) system comprises two IGFs (IGF-I and IGF-II), IGF-I and IGF-II receptors, and at least six distinct insulin-like growth factor binding proteins (IGFBPs) \(^{30}\). In the central nervous system, IGF-I and IGF-II support the growth and differentiation of neurons and glial cells \(^{3,12}\). The distribution of IGF-I and, IGF-II receptors have been studied in both rodent and human brains \(^{8,13,16,18,36}\). The IGF-I receptor is a heterotetrameric glycoprotein composed of two alpha and two beta subunits linked by disulphide bounds \(^{12}\). The IGF-II receptor is a monomeric receptor with a striking extracellular domain made up almost exclusively of 15 cysteine-based repeats, and is identical to the cation-independent mannose-6-phosphate receptor \(^{17,23}\).

IGFBPs play a crucial role in transporting IGFs in the circulation, cerebrospinal fluid, and across the capillary barrier to the target cells \(^{7,26}\). IGFBP-3 and IGFBP-5, in addition to binding IGFs, also can associate with an acid-labile subunit (ALS), thereby further increasing the half-life of the IGFs \(^{33,34}\). IGFBPs are also present at the level of the extracellular matrix or cell surface, where they can either enhance or inhibit the presentation of IGFs to their receptors \(^{10,14,25,30}\).

In addition, some of the IGFBPs, such as IGFBP-3, are also capable to mediate biological actions that are IGF independent \(^{10,25,30}\). More recently, a family of IGFBP-related proteins (IGFBP-rPs) has been identified that appear to have a 100-fold lower affinity for IGFs, compared with the IGFBPs. The functional roles of these IGFBP-rPs remain at present elusive \(^{14}\). This study was undertaken to characterise the cell membrane-bound binding sites for IGF-I in human brain and pituitary gland.

Material and methods

Materials

Human recombinant \(^{[125I]}\)IGF-I was obtained from Du Pont de Nemours (Dreieich, Germany). \(^{[125I]}\)standards and \(^{[3H]}\)Ultra films were obtained from Amersham Life Science (Amersham, Buckinghamshire, UK). IGF-I, DES(1-3)IGF-I and R\(^3\)-IGF-I were purchased from Gropep (Thebarton, Adelaide, Australia). DES(1-3)IGF-I is truncated IGF-I, missing the first 3 amino acids in the B domain, and R\(^3\)-IGF-I is an IGF-I analogue where glutamate at
position 3 is replaced by arginine. Recombinant human IGFBP-3 and the monoclonal antibody against the alpha-chain of human IGF-I receptor were purchased from Upstate (Lake Placid, NY, US). Biotinylated anti-chicken IgG was obtained from Immuno Jackson Chemicals (Baltimore, MD, USA). The polyclonal antibody against human IGFBP-3 was obtained from Gropep (Thebarton, Adelaide, Australia). The polyclonal antibody against human ALS was purchased from DSL (Webster, Texas, USA). Horseradish peroxidase (HRP)-streptavidin and HRP-conjugated goat-anti-rabbit were obtained from Bio-Rad (San Francisco, CA, USA). Normal rabbit serum was purchased from Zymed (San Francisco, CA, USA). The human hepatoma cell line HepG2 was a gift from D. Hoekstra (Rijksuniversiteit Groningen, Groningen, Netherlands). All cell culture plastic ware was obtained from Costar (Cambridge, MA, UK). DMEM (Dulbecco’s modified Eagle’s medium), antibiotics, all reagents for reverse transcription and all primers were from Life Technologies (Paisley, UK). Foetal calf serum was purchased from Bodinco (Alkmaar, The Netherlands). The High Fidelity PCR Master kit was obtained from Roche (Indianapolis, IN, USA) and the RNeasy® Mini Kit from Qiagen (Valencia, CA, USA). All other chemicals were of the highest grade commercially available.

Specificity of the antibodies

The antibody against human IGF-I receptor reacts specifically against a 19-residue synthetic peptide corresponding to amino acid residues (642-659) of the human IGF-I receptor alpha-subunit. The polyclonal IGFBP-3 antibody used in this study was obtained from rabbits immunised with a synthetic peptide of unique sequence from the central domain of human IGFBP-3. The specificity of the antibody was confirmed using the antibody preabsorbed with excess of matched recombinant human IGFBP-3. Specificity of the immunoreactivity was also controlled by the incubation of tissue sections in 5 % goat serum instead of primary antibodies; the immunohistochemical reactions were negative. For Western-blotting experiments, non-specific staining was determined by incubating parallel blots with non-immune rabbit serum instead of the primary antibody solution (Figure 4B). For immunoprecipitation experiments, non-specific binding was determined by incubating parallel blots with 3 % bovine serum albumin (BSA) instead of the primary antibody solution. The non-specific binding of the anterior pituitary is shown in Figure 5. The non-specific bands for serum and other brain samples were the same (not shown).
Tissues

Brains and pituitary glands were obtained from 10 patients without neurological or psychiatric diseases (five men and five women, ages between 54 and 78 years). Pituitary glands and 0.5-cm thick blocks of brain tissue were dissected at 0-4°C, frozen rapidly by immersion in isopentane-dry-ice, and stored at -80°C until used. Frontal cortex and white matter was obtained from the frontal gyri and cerebellum from one of the hemispheres. Care was taken to dissect comparable regions from the different individuals. The choroid plexus was obtained from the lateral ventricles. Post mortem delay, defined as the time elapsed between death and freezing of the tissue sections, ranged between 7 and 14 hours. An Ethical Committee approved the experiments on post-mortem brain.

[^125I] IGF-I binding studies

Frozen tissue blocks were mounted on a cryostat chuck, coated with embedding medium (OCT compound, Lab-Tek Products, Ontario, Canada) and serial sections, 10-µm thick, were cut at -20°C using a microtome-cryostat. Thereafter, sections were mounted on gelatine-coated glass slides, and dried overnight under vacuum. Sections were preincubated for 20 min at 20°C in 25 mM Tris/HCl (pH 7.4), containing 10 mM MgCl₂, and 0.1 % BSA. The optimal conditions for the binding experiments have been described previously 13. For competition experiments sections were incubated for 90 min with 0.1 nM [^125I]IGF-I and increasing concentrations of competitors IGF-I, DES(1-3)IGF-I, or R³-IGF-I. After incubation, the sections were washed three times for 1 min in the same buffer, to remove unbound ligand, and were then wiped from the slides using Whatman GF/B glass fiber filters. The level of radioactivity was determined in a gamma-counter. Binding isotherms were estimated by non-linear least-square curve fitting. The inhibition constants (KD values) of IGF-I, DES(1-3)IGF-I and R³-IGF-I were calculated by the method of Cheng and Prusoff ⁹. Autoradiographs were produced by incubating sections for 90 min at 20°C with 0.1 nM [^125I]IGF-I in the same buffer. Incubating consecutive sections with 0.5 µM IGF-I, or 0.5 µM DES(1-3)IGF-I, or 0.5 µM R³-IGF-I assessed non-specific binding. After incubation the sections were washed three times for 1 min in the same buffer and quickly dipped in distilled water. The sections were dried under a stream of cold air, placed in X-ray cassettes together with commercially available[^125I]-standards, and exposed to [³H]Ultra films for 4 days. The films were developed with a Kodak D19 developer at 4°C, and scanned with an Arcus plus
scanner. The images were digitised and analysed by computer-assisted densitometry using NIH image-analysis software. A calibration curve was generated by fitting of optical density and disintegration's per minute per milligram polymer values of the $^{125}\text{I}$ standards. The regions of interest were sampled and mean optical densities determined, and converted into fmol/mg protein, based on the experimentally determined relation between polymer and brain paste standards. Specific binding values were obtained by the subtraction of non-specific binding images from corresponding total binding images. All binding experiments were done in duplicate and repeated twice.

**Preparation of homogenates**

Frozen tissue blocks of approximately 1 g were homogenised in 10 ml ice-cold Tris/HCl buffer (25 mM, pH 7.4) containing serine-, cysteine-, aspartic- and metallo-proteinases inhibitors, with an Ultraturrax and Potter Elvehjem homogeniser, and centrifuged at 50,000 g for 15 min. The pellets, containing the cell membranes, were resuspended in 10 ml of the same buffer as indicated above and centrifuged twice at 50,000 g for 15 min. The final pellet was suspended in 1 ml of the same buffer containing 10 % glycerol (vol/vol), and stored at -80°C. Protein concentrations were determined by the method of Lowry et al. To be sure that there is no blood contamination from the circulation, albumin was measured in homogenates from the anterior and posterior pituitary gland by using an albumin immune assay (BN2, Behring, Gmbh, Marburg, Germany). In all the samples we used in this study the albumin fraction was not detectable.

**Western-immunoblotting and immunoprecipitation**

For Western-immunoblotting experiments the homogenates were diluted and resolved in sample buffer, and ran both under native conditions [no sodium dodecyl sulphate (SDS), no mercaptoethanol and no boiling], and reducing conditions (0.25 % mercaptoethanol, 0.5 % SDS and boiling during 6 min). Human IGFBP-3 was used as a positive control and diluted in sample buffer containing 0.1 % (w/v) SDS. Sixty micrograms of sample protein and 10 μl (0.001 μg/μl) human IGFBP-3 protein were loaded on the gel system and ran on 4-12 % SDS-polyacrylamide gel electrophoresis (PAGE).

For immunoprecipitation experiments 50 μl of the homogenates was rehomogenised in 200 μl ice-cold 50 mM Tris-buffer containing 6.25 mM EDTA, 1-% Triton-X-100 (pH 7.4) and the same protease inhibitors as indicated above. Homogenates were centrifuged at 3000 g for 10
min. Equal amounts of the supernatant (200 µl) were diluted in 200 µl detergent buffer [50 mM EDTA, 50 mM Tris pH 7.4, 0.2 % sodium deoxycholic acid (NaDOC), 0.05 % nonidet P-40 (NP-40), 0.5 % SDS]. The suspensions (400 µl), 100 µl human serum (positive control for ALS) and 10 µl human IGFBP-3 (0.001 µg/µl) were then incubated with 5 µg polyclonal human IGFBP-3 antibody for 24h at 4°C. The immunocomplexes were precipitated with agarose-G-beads at 4°C for 2h. After washing: three times with low-salt washing buffer (50 mM Tris-HCl, 0.05 % NaDOC, 0.1 % NP40, pH 7.4) and twice with phosphate buffered saline (PBS); the precipitated samples were resuspended in sample buffer containing 0.1 % SDS and boiled for 6 min. Fifty microlitres of the samples were loaded on the gel system and separated under reducing conditions using 4-12 % SDS-PAGE.

Proteins were transferred to nitrocellulose membranes and incubated overnight at 4°C with the primary antibody solution [chicken-anti-IGF-I receptor (1:500) or rabbit-anti-IGFBP-3 (1/1000) and or rabbit-anti-ALS diluted (1/1000)] in Tris buffered saline (TBS, pH 7.4) containing 0.5 % milk powder. For the detection of the IGF-I receptor, the membranes were incubated with biotinylated second antibody solution (rabbit-anti-chicken 1:600) in TBS containing 0.5 % milk powder, for 2 h at room temperature. Finally, membranes were incubated with HRP-conjugated streptavidin (1:600) for 2h at room temperature. For the detection of ALS and IGFBP-3, membranes were incubated with HRP-conjugated goat-anti-rabbit solution (1/1000) in TBS containing 0.5 % milk powder, for 2 h at room temperature. The complexes derived from Western-immunoblotting and immunoprecipitation experiments were visualised using freshly prepared diaminobenzidin (DAB, 30-mg/100 ml Tris buffer, pH 7.4 and 0.015 % H2O2), and the opti-4CN (4-chloro-1-naphtol) staining method of Bio-Rad. Between all steps the nitrocellulose membrane was rinsed in TBS for 30 min.

**Immunohistochemical experiments**

Frozen sections, 10-µm thick were preincubated in 25 mM Tris/HCl (pH 7.4) containing 10 mM MgCl₂. Sections that were and were not preincubated were fixated in 3 % buffered formaldehyde for 15 minutes and washed in PBS for 10 minutes. To exhaust endogenous peroxidase activity, sections were immersed for 10 min in 0.3 % H₂O₂ in PBS prior to the first antibody incubation. Before the addition of first and secondary antibody solution, sections were incubated for 30 min at room temperature in normal goat serum to suppress non-specific antibody binding. Thereafter, sections were incubated in primary antibody solution: rabbit-anti-IGFBP-3 (1:200) in PBS for 24 hr at 4°C. Sections were then incubated
with the secondary biotinylated antibody solution: goat-anti-rabbit (1:200) in PBS for 120
min at room temperature. Finally, sections were incubated in HRP-conjugated streptavidin
solution (1:200) in PBS for 90 min at room temperature and processed by the
diaminobenzidine/H₂O₂ reaction (30 mg DAB/100 ml Tris buffer, pH 7.4) and 0.01 % H₂O₂
and they were counterstained with 3% hematoxylin. Between all steps the sections were
rinsed thoroughly with PBS.

RT-PCR experiments

HepG2-cells were cultured in DMEM supplemented with 10% foetal calf serum, 50 U/ml
penicillin, and 50 µg/ml streptomycin in a humidified 5% CO₂ atmosphere at 37°C. Total
RNA was isolated from either frozen anterior pituitary tissue (100 mg/sample) or HepG2
cells (10⁷ cells/sample) using the RNeasy® Mini Kit and following the manufacturers
instructions. Single stranded cDNA was synthesised from 5 µg total RNA using 2.5 µg Oligo
d(T)₁₂⁻₁₈ primer, 1000 units Superscript™ RT II, 20 µl first strand buffer, 10 mM DTT
dithiotreitol) and 0.5 mM dNTP in a total volume of 100 µl. The RNA sample and the Oligo
d(T) primer were denatured at 65°C for 15 min and placed on ice for 5 min before addition to
the reaction tube. Reverse transcription was performed for 1 h at 37°C, and subsequently the
samples were heated to 99°C for 5 min to terminate the reaction. PCR was performed with
the High Fidelity PCR Master using 5 µl of the obtained cDNA and 7.5 pmol sense and
antisense primer, according to the manufacturers’ instructions. The final reaction volume was
50 µl. The tubes were incubated in a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk,
CT, USA) at 94°C for 5 min to denature the cDNA and primers. The cycling program was
94°C for 30 s, 55°C for 30 s, 72°C for 30 s for actin and 94°C for 30 s, 57°C for 30 s, 72°C
for 50 s (7 min in the last cycle) for ALS. For each primer set and sample, an increasing
number of PCR cycles (25 to 40) was performed with otherwise fixed conditions. The
following primers were employed: actin: 5’-AACACCCCGCCATGTAC-3’ (sense) and 5’-
ATGTACGCGACAGTTTCC-3’ (antisense, 254 bp amplified product) and ALS: 5’-
CTCAACCTCAGCTGGAATAG-3’ (sense) and 5’-CACAGGCTCTGCTCCTCAAT-3’
(antisense, 770 bp amplified product). In each experiment, water, RNA, and cDNA alone as
well as cDNA with only sense or antisense primer was used as a negative control to check for
contamination and specificity. Ten microlitres of PCR products were separated on 2.0%
agarose gels, stained with ethidium bromide, and photographed using a Polaroid DS34 Instant
Screen Direct Camera (Hertfordshire, UK).
Results

$[^{125}\text{I}] \text{IGF-I binding}$

We first studied the IGF-I binding sites in human brain and pituitary gland by using radioligand binding techniques with $[^{125}\text{I}]\text{IGF-I}$ and autoradiography. Before incubation with $[^{125}\text{I}]\text{IGF-I}$ tissue slices were preincubated in buffer to remove endogenous IGFs and other soluble proteins. On adjacent tissue sections of frontal cortex (Figure 1), cerebellum, choroid plexus, anterior and posterior pituitary, we performed $[^{125}\text{I}]\text{IGF-I}$ competition binding experiments with IGF-I, DES(1-3)IGF-I and R$^3$-IGF-I. Both, DES(1-3)IGF-I and R$^3$-IGF-I bind to IGF-I receptors but have low affinity for IGFBPs $^2, ^{31}, ^{32}$. In all brain regions and choroid plexus, $[^{125}\text{I}]\text{IGF-I}$ was equally displaced by IGF-I, DES(1-3)IGF-I, and R$^3$-IGF-I (Figure 1). The curves were best fitted to a single-site-binding model. The mean $K_i$ values ± SD were 1.0 ± 0.3 nM for IGF-I, 1.1 ± 0.2 nM for DES(1-3)IGF-I, and 1.2 ± 0.2 nM for R$^3$-IGF-I [$p = 0.74$ (Mann-Whitney)]. In contrast, in the anterior pituitary, there was no displacement of $[^{125}\text{I}]\text{IGF-I}$ binding with DES(1-3)IGF-I and R$^3$-IGF-I, and IGF-I had a 10-fold lower affinity [$K_i = 12.7 ± 1.5$ nM, $p<0.0001$ (Mann-Whitney)] for this binding site than in brain regions and choroid plexus (Figure 1).

We compared autoradiographs obtained following incubation with $[^{125}\text{I}]\text{IGF-I}$ in the absence and presence of 0.5 µM IGF-I, 0.5 µM DES(1-3)IGF-I, or 0.5 µM R$^3$-IGF-I. As shown in Figure 2, binding of $[^{125}\text{I}]\text{IGF-I}$ was equally displaced by all three competitors in cerebellum (not shown), frontal cortex, cerebral white matter, choroid plexus (not shown), and posterior pituitary. In the anterior pituitary (Figure 2), $[^{125}\text{I}]\text{IGF-I}$ binding was displaced by IGF-I, but not by DES(1-3)IGF-I or R$^3$-IGF-I, indicating that $[^{125}\text{I}]\text{IGF-I}$ labelled an IGF-binding site that was different from the IGF-I receptor. Non-specific binding of competition and autoradiographic experiments in the presence of IGF-I varied between 30 and 40 % of the total binding. $[^{125}\text{I}]\text{IGF-I}$ binding characteristics were not different between males and females. IGF-I receptor concentrations are shown in Table 1.
Representative competition curves for IGF-I (O), DES(1-3)IGF-I (▼) and R³-IGF-I (□) inhibition of 0.1 nM [¹²⁵I]IGF-I specific binding on microtome sections of human frontal cortex (A) and anterior pituitary (B). Computer analysis of the binding data revealed that the competition curves were best described by a one-component binding model. In frontal cortex [¹²⁵I]IGF-I was equally displaced by IGF-I, DES(1-3)IGF-I and R³-IGF-I. Ki-values were 0.9 nM for IGF-I and DES(1-3)IGF-I, and 1.0 nM for R³-IGF-I. In the anterior pituitary [¹²⁵I]IGF-I was displaced by IGF-I (Ki-value of 12 nM) but not by DES(1-3)IGF-I and R³-IGF-I.
Table 1  IGF-I receptor concentrations labelled by [125I]IGF-I in human adult brain samples and pituitary gland (values are expressed in fmol/mg).

<table>
<thead>
<tr>
<th>Region</th>
<th>males (n=5)</th>
<th>females (n=5)</th>
<th>*p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex</td>
<td>24.6 ± 2.7</td>
<td>23.6 ± 3.7</td>
<td>0.84</td>
</tr>
<tr>
<td>Frontal white matter</td>
<td>8.4 ± 1.8</td>
<td>9.4 ± 2.7</td>
<td>0.69</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granule cell layer</td>
<td>12.6 ± 2.1</td>
<td>13.8 ± 4.2</td>
<td>0.84</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>36.4 ± 5.7</td>
<td>38.2 ± 6.7</td>
<td>0.42</td>
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<tr>
<td>Deep white matter</td>
<td>9.0 ± 1.8</td>
<td>10.7 ± 3.0</td>
<td>0.39</td>
</tr>
<tr>
<td>Dendate nucleus</td>
<td>17.6 ± 2.7</td>
<td>20.0 ± 5.3</td>
<td>0.42</td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>46.4 ± 8.1</td>
<td>43.4 ± 4.9</td>
<td>0.42</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior lobe</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Posterior lobe</td>
<td>23.8 ± 3.6</td>
<td>21.6 ± 2.7</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Slide mounted tissue sections were incubated with 0.1 nM [125I]IGF-I. Autoradiograms were generated by exposing the slide-mounted tissue sections to [3H]Ultratfilm for 4 days, and subsequently quantified by computerised densitometry. Non-specific binding was determined in the presence of DES(1-3)IGF-I and or R3-IGF-I. ND; not detected. Data are the mean ± SD. * = Mann-Whitney U-test.

IGF-I receptor and IGFBP-3-binding in homogenates from human pituitary and brain regions

To further examine the nature of the IGF-I binding site in the anterior pituitary, we performed western-immunoblot analysis and immunoprecipitation using a monoclonal antibody against the human IGF-I receptor, and polyclonal antibodies against human IGFBP-3 and ALS. The antibody against human IGF-I receptor reacts specifically against a 19 residue synthetic peptide corresponding to amino acid residues (642-659) of the human IGF-I receptor alpha-subunit. In frontal cortex, choroid plexus, cerebellum, white matter, and posterior pituitary a double band of 118-kDa corresponding to the alpha-chains of the IGF-I receptor were identified (Figure 3). In the anterior lobe of the pituitary gland no reaction with anti-human IGF-I receptor was found (Figure 3).
Figure 2

Autoradiographs of 0.1 nM $[^{125}\text{I}]$IGF-I binding in the absence = total binding (i) and the presence of 0.5 µM unlabeled IGF-I = non-specific binding (ii), or 0.5 µM DES(1-3)IGF-I (iii), or 0.5 µM R$^3$-IGF-I (iv) to microscope slide-mounted sections of frontal cortex (A) and pituitary gland (B). In frontal cortex (co), white matter (wm), and posterior pituitary (pp), $[^{125}\text{I}]$IGF-I was equally displaced with IGF-I, DES(1-3)IGF-I and R$^3$-IGF-I. In the anterior pituitary (ap), $[^{125}\text{I}]$IGF-I was displaced with IGF-I, but not with DES(1-3)IGF-I and R$^3$-IGF-I. Scale bar = 1 cm.
Western-immunoblots of brain samples and pituitary samples ran on 7.5 % SDS-PAGE under reducing conditions using an antibody against the alpha-chains of the human IGF-I receptor. Bands were visualised using DAB. We found two bands of approximately 118-kDa corresponding to the alpha-subunits of IGF-I receptor into detergent-solubilised total cell membrane fraction (60 µg) of frontal cortex (A), choroid plexus (B), cerebellum (C), posterior pituitary gland (D) and cerebral white matter (F). We found no expression of IGF-I receptor alpha-subunits in pellet fractions of the anterior pituitary (E).

Western blotting was performed under different conditions. In the first approach pellet fractions of the anterior pituitary ran under denaturing and reducing conditions, and were stained for IGFBP-3. A single band of 40-kDa (Figure 4A) was detected under these conditions. When the same samples ran under native conditions, and were stained for IGFBP-3 they revealed one band of 150-kDa (Figure 4A). Purified IGFBP-3 was used as a positive control. With anti-IGFBP-3 we found one band of 40-kDa (Figure 4A). Non-immune rabbit serum was used to determine the non-specific binding (Figure 4B).

We performed immunoprecipitation experiments to visualise the components of the complex. Immunocomplexes of proteins with anti-IGFBP-3, revealed a band of approximately 40-kDa in anterior pituitary, posterior pituitary and frontal cortex, just as human IGFBP-3 that was used as a positive control (Figure 5). Homogenates of the anterior pituitary immunoprecipitated with anti-IGFBP-3 and stained with anti-ALS revealed an 86-kDa band. The same band was present in human serum that contains ALS and was used as a positive control. This was not observed in the frontal cortex and the posterior pituitary (Figure 5).
Thus these results showed the presence of an IGFBP-3-ALS complex in the anterior pituitary. This complex was absent in the posterior pituitary and frontal cortex.

Figure 4

Western-immunoblots of anterior pituitary samples ran on 12 % SDS-PAGE both under native conditions, and under reducing and denatured conditions. Samples were stained for IGFBP-3 (A). Samples ran under native conditions revealed a 150-kDa-band (i) and samples that ran under reducing conditions revealed a single band of 40-kDa (ii). Human IGFBP-3 was used as a positive control and presents one band of 40-kDa (iii). In figure B, non-specific binding was determined by incubating parallel blots with non-immune rabbit serum instead of anti-IGFBP-3. Samples ran under native conditions (i), denatured conditions (ii), and pure IGFBP-3 (iii) didn’t reveal non-specific bands. Protein marker is shown at the left. Bands were visualised using opti-4CN.
Figure 5

4-12 % SDS-PAGE

Immunoprecipitation of proteins with anti-IGFBP-3 in anterior pituitary (ap), posterior pituitary (pp), frontal cortex (co), human IGFBP-3 (bp3) and serum (ser). Bands were visualised using opti-4-CN. Immunocomplexes stained for IGFBP-3 (A) showed one band of 40-kDa in anterior pituitary (ap), posterior pituitary (pp), frontal cortex (co) and for IGFBP-3 used as a positive control (BP3). Immunocomplexes stained for ALS (B) showed a single band of 86-kDa in anterior pituitary (ap) and serum (ser), but not in the posterior pituitary (pp) and frontal cortex (co). Non-specific bands (nsb) of the anterior pituitary are shown using BSA instead of the primary antibody.

IGFBP-3 binding to cell membranes

To confirm the presence of IGFBP-3 in the anterior pituitary, we performed immunohistochemistry both with and without preincubation in 25 mM Tris-buffer supplemented with 10 mM MgCl₂, using a polyclonal antibody against IGFBP-3. Without preincubation, IGFBP-3 was present in the anterior and posterior pituitary, and in the frontal cortex (Figure 6). IGFBP-3 was located in anterior pituitary cells and in the posterior pituitary, IGFBP-3 was located in the pituicytes. In the brain regions, IGFBP-3 was located in neurons. Following preincubation in 25 mM Tris-buffer supplemented with 10 mM MgCl₂; IGFBP-3 was no longer detectable in the posterior pituitary and any of the brain regions, but was still present in the anterior pituitary.
Photomicrographs of IGFBP-3 immunohistochemistry in anterior (A, B) and posterior pituitary gland (C, D) and frontal cortex (E, F). Nuclei are counterstained with hematoxylin (dark blue). Prior to preincubation in Tris-buffer containing MgCl₂, IGFBP 3 was present on cells in anterior (A) and posterior pituitary (C), and on neurons in frontal cortex (E). After preincubation, expression of IGFBP-3 was no longer detectable in posterior pituitary (D) and in frontal cortex (F). In contrast, IGFBP-3 was still present on cells in anterior pituitary (B). Scale bars = 100 µm.
**ALS mRNA expression in anterior pituitary**

In order to prove whether the ALS protein found in the anterior pituitary derives from the anterior pituitary itself or from the circulation, we performed RT-PCR on frozen anterior pituitary tissue of three patients. To date, it has not been shown that blood cells produce ALS. However, as it is not possible to wash tissue extensively before RNA isolation, we also tested samples of freshly isolated blood cells to exclude any possibility that mRNA of blood cells may falsify the results. The human hepatoma cell line HepG2 was used a positive control as it is known that ALS is highly expressed in liver. Furthermore, actin mRNA levels were used to standardise RNA contents of different samples. These results show that ALS mRNA is detectable in the positive control HepG2 cells and the anterior pituitary (Figure 7). When comparing the relative band intensities, the ALS mRNA level in the anterior pituitary ranged between 20 and 30% of the level in HepG2 cells. No specific signal for the ALS mRNA fragment was detected in the blood cell samples. The bands seen in blood cell samples are of smaller size and therefore, unspecific. This rules out that the ALS mRNA in the anterior pituitary derives from contamination with blood.

![Figure 7](image)

RT-PCR was performed with RNA isolated from anterior pituitary tissue of three patients, blood cells and HepG2 cells. The PCR products were separated on a 2% agarose gel. One representative ethidium bromide-stained gel is shown. The amplified ALS fragment (770 bp) was detected in HepG2 cells (lane 1 and 2) and anterior pituitary tissue (lane 3 and 4) after 35 and 40 cycles but not in whole blood (lane 5 and 6). Comparable amounts of the amplified actin fragment (254 bp, internal control) were detected in HepG2 cells (lane 7), anterior pituitary tissue (lane 8), and whole blood (lane 9) after 35 cycles. Names of the genes are indicated on the left and the corresponding fragment sizes on the right. H: HepG2 cells, p: anterior pituitary, b: blood cells.
Discussion

IGF-I binding sites are widely distributed throughout human brain and pituitary gland\textsuperscript{13}. The brain contains not only IGF receptors but also the six different IGFBPs that have been identified\textsuperscript{25, 30}. These IGFBPs are soluble proteins, and we showed that preincubation in a buffer solution (Tris/HCl containing 10 mM MgCl\textsubscript{2}) readily abolished both the autoradiographic labelling and immunohistochemical visualisation of IGFBPs in slices of different brain regions\textsuperscript{36}. The binding characteristics of \textsuperscript{125}IIGF-I in the brain regions and posterior pituitary were compatible with binding to IGF-I receptors.

In the anterior pituitary, \textsuperscript{125}IIGF-I binding occurred to a binding site with a 10-fold lower affinity than the IGF-I receptor. Using western-immunoblotting experiments with anti-IGFBP-3 under native conditions, we identified this binding site as a 150 kDa-IGFBP-3-containing molecule. In this region, preincubation in buffer did not abolish IGFBP-3 immunoreactivity, indicating that this IGFBP-3-containing complex was tightly bound to the cell membranes. Additionally to these findings IGFBP-3 was completely complexed with ALS because we found no smaller proteins with anti-IGFBP-3. In contrast, when the same samples were analysed under high reducing and denaturing conditions, we identified a single 40-kDa IGFBP-3 band. Under these conditions the 150-kDa complex was dissociated.

Immunoprecipitation experiments confirmed the presence of a 150-kDa complex in the anterior pituitary. When proteins of the anterior pituitary were complexed with anti-IGFBP-3 and these immunocomplexes were stained for IGFBP-3, they showed one band of approximately 40-kDa, and when stained for ALS they showed an 86-kDa band. Immunocomplexes from the posterior pituitary and frontal cortex, which were stained for ALS, did not reveal an 86-kDa band, indicating the absence of ALS. When these immunocomplexes were stained for IGFBP-3, they revealed a band of approximately 40-kDa, indicating the presence of IGFBP-3 in both anterior and posterior pituitary and frontal cortex.

It has been shown previously that IGFBP-3 has cellular effects that are independent of IGF presence or action, and these effects are mediated by binding of IGFBP-3 to the cell surface, possibly to specific receptors\textsuperscript{22, 25, 38}. However, since IGF-I in association with IGFBP-3 prevents binding of IGFBP-3 to the cell membrane\textsuperscript{29}, the IGFBP-3 binding site in the anterior pituitary must be of a different composition.
The molecular weight of IGFBP-3 in its non-glycosylated form is 29-kDa, in its glycosylated state 40 - 44-kDa, and when it forms a complex with ALS, 140 - 150-kDa. IGFBP-3 is the predominant carrier protein of IGF-I in serum. The IGF-I:IGFBP-3 dimer forms a complex with ALS, and this ternary complex prolongs the serum half-life of IGF-I by many hours. Once released from the complex, IGF-I leaves the circulation and enters target tissues with the aid of IGFBPs present on the cell surface or in the extracellular matrix.

By using RT-PCR in anterior pituitary and blood samples, we found the expression of ALS mRNA in the anterior pituitary and not in blood samples. Thus, our findings suggest that the human anterior pituitary gland expressed ALS mRNA and ALS protein and these ALS forms a complex with IGFBP-3 bound to the cell membrane. ALS is mainly synthesised by the liver and the native ALS molecule appears in serum as an 84 - 86-kDa glycoprotein doublet. Interestingly, it has been shown that follicular fluid ALS originates from the ovary and that ALS is also synthesised by the kidney.

We were unable to detect IGF-I receptors in the anterior pituitary gland in this study. However, our in situ observations do not entirely exclude their presence in vivo. IGF-I receptor mRNA has been demonstrated by in situ hybridisation in rodent anterior pituitary slices and cell cultures. It might be possible that the receptors were down regulated by IGF-I, as has been shown for IGF-I receptors in rat pituitary cells.

In conclusion, our findings show that in human anterior pituitary gland IGF-I binds to a not previously reported tightly membrane-bound IGFBP-3-ALS complex. The functional implication of the binding of IGF-I to this complex requires further experimentation at the cellular level.
References


