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DEMONSTRATION AND STRUCTURAL COMPARISON OF RECEPTORS FOR INSULIN-LIKE GROWTH FACTOR-I AND -II (IGF-I AND -II) IN BRAIN AND BLOOD-BRAIN BARRIER

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SUMMARY: Specific receptors for insulin-like growth factors (IGF) I and II on microvessel-free rat brain cell membranes (RBCM) and in the microvessels that constitute the blood-brain barrier (BBB) were identified and characterized by means of affinity cross-linking techniques and specific anti-receptor antibodies. Two different models of BBB were examined: isolated rat brain capillaries and cultured bovine brain microvessel endothelial cells. Cross-linking with 125-I-IGF-I, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), revealed an α subunit of apparent M_r = 138,000 in both BBB preparations, compared to 120,000 in RBCM. Cross-linking was inhibited by unlabeled IGF and insulin, but not by antibody directed against the IGF-II receptor. When 125-I-IGF-II was cross-linked, followed by SDS-PAGE under reducing conditions, a major band of apparent M_r = 250,000 was identified in RBCM and both BBB preparations. This band, which migrated with an approximately equivalent Mr in both brain and BBB membranes, was inhibited by unlabeled IGF and by antibody specific for the IGF-II receptor. Thus, both rat and bovine brain microvessels possess classical Type I and II IGF receptors. While the α subunit of the Type I receptor of brain is smaller than that of the BBB, the Type II receptor of brain and BBB appear to be structurally and immunologically identical.

Recent investigations have led to the identification and characterization of specific receptors for insulin (1-8) and the insulin-like growth factors (IGFs) (9-17) in the central nervous system (CNS). As is the case in peripheral tissues, CNS receptors for both insulin and IGF-I are heterotetramers, comprised of two α and two β subunits (18). However, the α subunits of CNS insulin and IGF-I receptors migrate on SDS-PAGE with an apparent M_r 10-20,000 less than the peripheral α subunits (1,3,5,6, 10,15,17). This apparent decrease in size of CNS insulin and IGF-

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I receptor α subunits is believed to result from differences in carbohydrate composition (3,5,17,19). On the other hand, preliminary structural characterization of the IGF-II receptor in rat brain cortical plasma membranes, recently reported by Gammeltoft et al (10), demonstrates a M_r = 250,000 monomer, similar to that found in rat liver.

The source of both IGF-I and IGF-II in the CNS remains a subject of some controversy. Synthesis of IGFs by cultured brain explants has been demonstrated (20), and high levels of IGF-II in human cerebrospinal fluid (21,22) and brain (23) have been reported. Frank et al (24), however, have recently demonstrated specific high-affinity receptors for insulin, IGF-I and IGF-II on bovine brain capillaries, suggesting that selective transport of insulin and the IGFs across the blood-brain barrier (BBB) and into the CNS is a distinct possibility.

The investigations described below have employed affinity cross-linking techniques and specific anti-receptor antibodies to compare receptors for IGF-I and IGF-II in microvessel-free brain cell membranes and in the microvessels that constitute the BBB. Because brain capillaries are comprised of at least two types of cells (endothelial cells and pericytes), we have examined two different BBB models: isolated rat brain capillaries and cultured bovine brain microvessel endothelial cells.

MATERIALS AND METHODS

Peptides: Pure biosynthetic (Thr-59)-IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA). Pure synthetic IGF-II was generously provided by Dr. C. H. Li (San Francisco, CA). Recombinant DNA-derived human insulin was obtained from Eli Lilly & Company (Indianapolis, IN). Iodination was performed by a modification of the chloramine-T technique, to specific activities of 150-250 uCi/ug for both (Thr-59)-IGF-I and IGF-II. For determination of nonspecific binding, we employed a partially purified, insulin-free IGF preparation, containing 10 ug/mg weight IGF-II and 20 ug/mg weight IGF-I.

Anti-IGF-II Receptor Antibody: A polyclonal antibody generated against the rat IGF-II receptor (R-II-PAB1) was employed (25). This antibody both immunoprecipitates the type II IGF receptor, and specifically inhibits IGF-II binding to a variety of rat tissues. Immune serum was precipitated in 45% saturated ammonium sulfate, resuspended in PBS, and dialyzed against PBS.

Isolation of Rat Brain Microvessel Plasma Membranes: Rat brain cortical capillaries were isolated with a mechanical homogenization technique from 50 adult male Sprague-Dawley rats (200-300g), as previously described (26). These microvessels are free of adjoining brain tissue when examined by light or phase microscopy, and consist of endothelial cells and pericytes in an

approximate 3:1 ratio. Brain capillaries were subsequently subjected to hypotonic lysis, and the microvessel ghosts collected as a pellet after a 15,000g, 10 minute centrifugation at 4C. The capillary plasma membrane was separated from the basement membrane as previously described (26), and stored at -20C. Protein determinations were performed by the modified Bradford assay (BioRad, Richmond, CA). Rat blood-brain barrier membrane preparations will be abbreviated RBBB.

Preparation of Rat Brain Cell Membranes (RBCM): Synaptosomal membranes were isolated by the method of Gray and Whittaker (27), as previously described (28), and shown to be 98% free of capillary membranes. Membrane preparations were stored at -20C.

Culture of Bovine Brain Capillary Endothelial Cells: Bovine microvessel endothelial cells were isolated by the method of Bowman et al (29), as modified by Audus and Borchardt (30). Cells were cultured in equal parts of F-12 Ham nutrient mix (Hazelton Research Products, Lenexa, Kans.) and minimal essential medium, 10 mM Hepes, 13 mM sodium bicarbonate, pH 7.4, 100 ug/ml penicillin-G, 100 ug/ml streptomycin, 50 ug/ml polymyxin B, 2.5 ug/ml amphoterecin B, 100 ug/ml heparin (Sigma Chemical Co., St. Louis, Mo.), and 10% plasma-derived horse serum (HyClone Laboratories, Logan, Utah). All growth surfaces were coated with rat-tail collagen prepared and applied by the method of Michelopoulis and Pitot (31), followed by treatment with 10 ug/cm bovine fibronectin (ICN Biochemicals, Cleveland, OH) for 1 hr.

Preparation of Bovine Endothelial Cell Membranes (Bovine Blood-Brain Barrier: BBBB): Crude membrane preparations from confluent bovine brain microvessel endothelial cells were prepared, as described above, and stored at -20C.

Affinity Cross-Linking: Membranes were resuspended in 50 mM Tris HCl, 0.5% bovine serum albumin, pH 7.4. For each gel lane 100 ug (RBBB and RBCM) or 200 ug (BBBB) membrane protein were incubated with 750,000 cpm iodinated peptide in the presence and absence of unlabeled peptide for two hours at 22C, followed by 30 minutes at 4C. For those lanes where receptor antibody was employed, membranes were preincubated with R-II-PAB1 (200 ug/ml protein) for one hour at 22C prior to addition of radioligand. After washing, the membrane pellet was resuspended in 1 ml of 50 mM Tris HCl, pH 7.4, and the radioligand cross-linked to receptor with 0.2 mM disuccinimidyl suberate in a volume of 1 ml, at 4C for 15 minutes. The reaction was quenched by the addition of 200 ul of 50 mM Tris HCl, 5 mM EDTA, pH 7.4. The pellets were solubilized in 100 ul of 2% SDS, pH 7.0, with 10% glycerol and 0.01% bromphenol blue, and electrophoresed on a 6% separating gel in the presence of 5% v/v 2-mercaptoethanol.

RESULTS AND DISCUSSION: Figure 1 shows the results of SDS-PAGE of 125-I-IGF-I cross-linked to RBBB, RBCM, and BBBB, and run under reducing conditions. In membrane preparations from both RBBB (lanes 1-4) and BBBB (lanes 9-12), two major bands are observed. The first migrates at apparent M_r = 138,000 and is totally inhibited by excess IGF, partially inhibited by insulin, and not inhibited by R-II-PAB1, indicating that this band

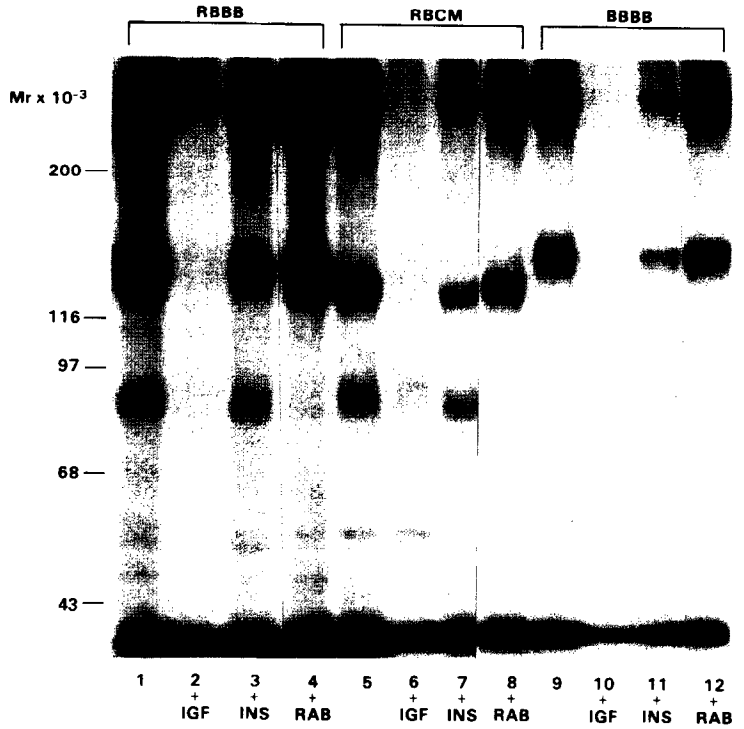

Figure 1. SDS-PAGE of 125-I-IGF-I cross-linked to membrane preparations derived from rat blood brain barruer (RBBB, lanes 1-4), rat brain cell membranes (RBCM, lanes 5-8), and bovine blood brain barruer (BBBB, lanes 9-12), as described in Materials and Methods. Incubations of membranes with 125-I-IGF-I were performed in the absence of competing peptides (lanes 1, 5, 9), or in the presence of excess unlabeled IGF (2 ug/ml IGF-I + 1 ug/ml IGF-II; lanes 2, 6, 10), unlabeled insulin (INS: 100 ug/ml; lanes 3, 7, 11), or anti-receptor antibody R-II-PAB1 (RAB: 100 ug/ml; lanes 4, 8, 12).

represents the α subunit of the Type I IGF receptor. The second major band migrates with an apparent M_r = 250,000. Like the M_r = 138,000 band, it is totally inhibited by excess IGF, partially inhibited by insulin, and not inhibited by R-II-PAB1, suggesting that it represents an incompletely reduced $\alpha-\alpha$ dimer, rather than 125-I-IGF-I cross-linked to the Type II IGF receptor. Minor bands are also observed at apparent Mr = 85,000 and M_r = 45,000. These bands are inhibited by IGF, but not by insulin, and may represent 125-I-IGF-I cross-linked to binding protein or to degradation products of the Type I or II IGF receptor.

The same bands appear on SDS-PAGE of RBCM (lanes 5-8), but now migrate at apparent M_r = 120,000 and 240,000. The smaller band (M_r = 120,000), like its counterpart in RBBB and BBBB, is totally inhibited by excess IGF, partially inhibited by insulin, and not inhibited by R-II-PAB1. Presumably, it represents a lower

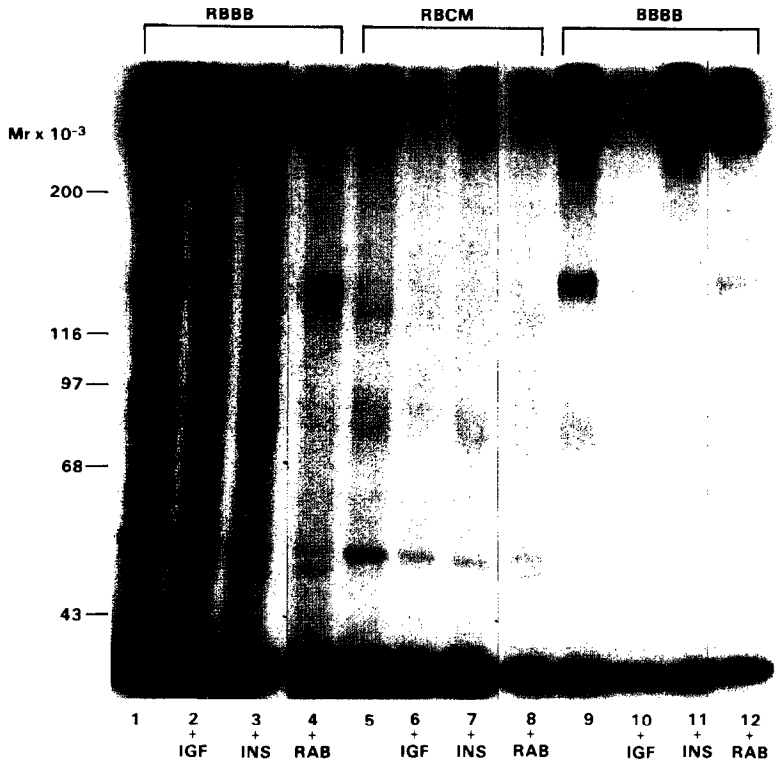

Figure 2. SDS-PAGE of 125-I-IGF-II cross-linked to membrane preparations derived from rat blood brain barrier (RBBB, lanes 1-4), rat brain cell membranes (RBCM, lanes 5-8), and bovine blood brain barrier (BBBB, lanes 9-12), as described in Materials and Methods. Incubations of membranes with 125-I-IGF-II were performed in the absence of competing peptides (lanes 1, 5 , 9), or in the presence if excess unlabeled IGF (2 ug/ml IGF-I + 1 ug/ml IGF-II); lanes 2, 6, 10), unlabeled insulin (INS: 100 ug/ml; lanes 3, 7, 11), or anti-receptor antibody R-II-PAB1 (RAB: 100 ug/ml; lanes 4, 8, 12).

molecular weight form of the α subunit of the Type I IGF receptor. The larger band represents an α-α dimer, since it is inhibited by unlabeled IGF and insulin, but not by R-II-PAB1.

Figure 2 shows affinity cross-linking of 125-I-IGF-II. A major band is observed at apparent M_r = 250,000 in RBBB, RBCM, and BBBB. Like the M_r = 250,000 band seen during 125-I-IGF-I cross-linking, this band is completely inhibited by excess IGF. However, it is not inhibited at all by insulin, while it is clearly inhibited by R-II-PAB1, indicating that this is not the Type I receptor (or a dimer of its subunits), but rather, represents the Type II IGF receptor cross-linked to 125-I-IGF-II. It is of note that, unlike the 120-138,000 and 240-250,000 bands seen on the previous gel, there is no observable difference

between the apparent M_r of these high molecular weight bands in RBBB, RBCM and BBBB. The apparent minor increase in M_r of this band in lane 4 is apparently artifactual, since it has not been seen in any other gels. It is important to recognize, however, that the discriminatory capability observed at the top of a 6% separating gel is limited, and that we may be missing small differences in relative molecular mass (in the order of M_r = 5000). A fainter band is observed at apparent M_r = 138,000 in RBBB and BBBB, and M_r = 120,000 in RBCM. As in the previous gel, this band is inhibited by excess IGF, but not by insulin or R-II-PAB1, indicating that this represents 125-I-IGF-II cross-linked to the Type I IGF receptor.

These studies thus demonstrate Type I and II IGF receptors in microvessel-free rat brain cortical membranes, as well as two distinct models of BBB cells. In rat brain, the α subunit of the Type I receptor migrates with a M_r = 120,000 (M_r = 113,000 if the MW of IGF-I is subtracted). These findings are similar to the reports by Gammeltoft et al (cross-linked α subunit: M_r = 115,000) (10) and Heidenreich et al (M_r = 120,000) (17) in rat and human brain membranes, respectively, as well as the recent description of a M_r = 122,500 cross-linked α subunit in cultured rat brain neurons (15).

The data presented above permit the first comparison of IGF receptors in BBB membranes and BBB-free brain cell membranes. It is clear that the size of the rat brain α subunit is different from that observed in both rat and bovine BBB. Cross-linking of IGF-I to membrane preparations derived from rat brain microvessels and bovine brain capillary endothelial cells demonstrates an α subunit with an apparent M_r = 138,000. This is similar to the M_r = 133,000 band reported by Frank et al (24) in isolated bovine brain capillaries. Thus, brain microvessels have classic Type I IGF receptors, which are similar to those reported in peripheral tissues.

These studies also directly demonstrate that the Type II receptor in the CNS is a monomer with an apparent Mr similar to that observed in brain microvessels and peripheral tissues. This M_r = 250,000 band is inhibited by excess unlabeled IGF, but not at all by insulin. Furthermore, the band is clearly also inhibited by antibody against the Type II receptor (particularly in rat tissues), thus distinguishing it from the 240,000-250,000 dalton band observed with cross-linking of 125-I-IGF-I.

The size difference between the α subunits of the Type I receptor in CNS and BBB is presumably due to variations in carbohydrate composition, as demonstrated for the insulin receptor (1,3,5,6) . Heidenreich et al (19) have shown that the size discrepancy between human placental and brain IGF-I receptors disappears after the former are treated with either neuraminidase or endo-N-acetylglucosaminidase. Whether the altered structure of CNS IGF-I receptors (or the apparently unaltered structure of CNS IGF-II receptors) is involved in special biologic activities of these peptides in the brain remains to be demonstrated. Similarly, how receptor structure influences the ability of BBB receptors to selectively transport insulin and/or the IGFs into the CNS awaits further investigations.

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