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Abnormal serum IGF-II transport in non-islet cell tumor hypoglycemia results from abnormalities of both IGF binding protein-3 and acid labile subunit and leads to elevation of serum free IGF-II.

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The syndrome of non-islet cell tumor hypoglycemia (NICTH) is the result of hypersecretion of IGF-II by a tumor although serum IGF-II is seldom elevated. This is attributable to abnormalities of the IGF binding proteins (IGFBPs) present in NICTH which is characterized by a marked decrease in the fraction of IGFBP-3 present in the 150 kD complex with acid labile subunit (ALS) and a 2- to 4-fold increase in IGFBP-2. We studied the impact of these changes in IGFBPs on the concentration of free IGF-II using a neutral C-18 Sep-Pak extraction procedure. We found that free IGF-II was increased 8- to 20-fold in NICTH. Thus there is no limitation of free IGF-II for complex formation. Additional experiments were conducted to determfine whether ALS deficiency limits 150 kD complex formation. We observed that addition of purified ALS to NICTH sera only partially succeeded in converting smaller complexes containing IGFBP-3 to large 150 kD complexes. We conclude that both a functional deficiency of ALS and IGFBP-3 are present in NICTH sera. The increased free IGF-II in NICTH sera contributes greatly to bioactivity and largely explains the marked hypoglycemia of NICTH patients even when total serum IGF-II concentrations may remain within normal limits.

Keywords: Tumor hypoglycemia; IGF binding proteins; IGF-II; Free-IGF-II

Introduction

It is now generally agreed that the syndrome of non-islet cell hypoglycemia (NICTH) in most cases is the result of hypersecretion of IGF-II by the tumor. The failure to find an increase in the total concentration of IGF-II in this condition (Widmer *et al.*, 1982) is attributable to abnormal binding of IGF-II by insulin-like growth factor binding proteins (IGFBPs). Normally about 80% of the IGF-I and II is present in serum complexed to IGFBP-3 in equimolar concentrations with an acid labile subunit (ALS) to form a 150 kD complex (Baxter & Martin, 1989). In NICTH the 150 kD complex is markedly decreased. (Baxter & Daughaday, 1991; Zapf *et al.*, 1992; Fukuda *et al.*, 1993). As the 150 kD complex is largely restricted to the circulation the predominance of small complexes allows greater passage through the capillary wall and access of the transported IGF-II to target tissue receptors.

The reason for the defective formation of the 150 kD complex is not clear. Baxter & Daughaday (1991) found that the concentrations of IGFBP-3 and ALS to be reduced about 50% in NICTH serum but did not consider this to be responsible for the abnormality. To gain further information about the cause of the defective formation of the 150 kD complex we and others have added the separate components of the ternary complex to NICTH serum to determine which could restore the normal pattern of IGF serum binding. When bound ^{125}I IGF-II covalently linked to IGFBP-3 was added to NICTH serum the 150 kD complex was formed suggesting that there was sufficient ALS in NICTH serum to form the 150 kD complex. However, when Zapf *et al.* (1992) added ^{125}I IGFBP-3 to NICTH serum it failed to enter the 150 kD complex suggesting that the defect lay in a deficiency of ALS. To resolve these conflicting data we report the results of experiments in which we have added purified ALS to normal and NICTH sera and compared the reconstitution of the 150 kD complex.

Much of the IGF-II immunoactivity present in NICTH sera is present as partially processed 'big' IGF-II (Daughaday & Kapadia, 1989). Qualitative or quantitative abnormalities of big IGF-II do not appear to be responsible for the abnormal IGF binding present in NICTH sera. There appears to be no abnormality in the ability of 'big' IGF-II extracted from NICTH serum or synthesized by recombinant methods to bind to either purified or recombinant IGFBP-3 (Zapf *et al*, 1992; Daughaday *et al.*, 1993). In addition when 'big' IGF-II partially purified from NICTH serum or recombinant proIGF-II (E1-21) were incubated with ^{125}I ALS and purified IGFBP-3 the ternary complex was immunoprecipitated.

The suggestion has been made that sequestration of big IGF-II into complexes less than 60 kD could account for the decrease in 150 kD complexes (Zapf *et al.*, 1992). A 2- to 6-fold increase in IGFBP-2 occurs in NICTH serum and this might compete with IGFBP-3 for IGF-II binding (Zapf *et al.* 1992; Blum *et al.*, 1993). This suggestion implies that the concentration of free IGF-II in NICTH serum is reduced. In this paper we describe measurements of free IGF-II with a neutral Sep Pak extraction method developed by Hizuka *et al.* (1991) for IGF-I which show that free IGF-II by this method is markedly increased in NICTH serum.

Results

To determine whether we could reproduce the results of Hizuka *et al.* (1991) in our laboratory we measured the free IGF-I in the sera of nine normal adult subjects. We found 2.9 ± 0.5 (SD) ng/L (0.38 ± 0.15 nMol/L). These results were only slightly higher than reported by Hizuka *et al.* of 2.4 ± 0.5 ng/L.

We next measured free IGF-II with this method. We established the consistency of the extraction by preparing five Sep-Pak extracts of a serum from a patient with NICTH. The free IGF-II of these separate extracts was 11.4 ± 1.6 (SD) nMol/L. On another occasion we extracted seven different sera of a patient with NICTH during an infusion of somatostatin. As no effect occurred the results provide a measurement of variance of repeated sampling from the same patient. The concentration of free IGF-II was 15.7 ± 4.8 (SD) nMol/L. The results of measurements of free IGF-II in sera of normal and pathologic states are presented in Figure 1. The mean concentration of free IGF-II in the sera of 17 normal adults was 1.77 ± 0.64 (SD) nMol/L. The mean total IGF-II was measured in 8 of these sera and found to be 111 ± 41 (SD) nMol/L and the per cent free was 1.82 ± 0.89.

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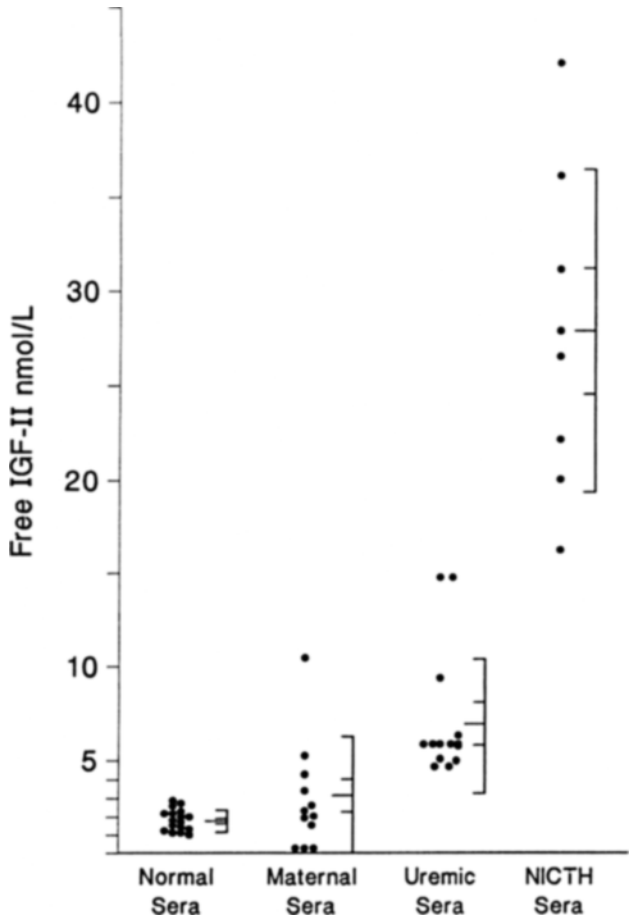

Figure 1 Free IGF-II was measured in sera fron non-gravid adults, from pregnant women at term, and from patients with uremia and NICTH

Because of the partial proteolysis of IGFBP-3 which occurs in the sera of women during pregnancy we studied the free IGF-II concentration in their sera. The mean free IGF-II in 12 sera obtained at term was 2.9 ± 0.8 nMol/L which was not significantly different from the results which we had obtained in men and non-gravid women.

A decrease in the proportion of IGFs carrried in the 150 kD complexes and other IGFBP abnormalities occurs in uremia. We measured free IGF-II in sera from 13 patients who were receiving chronic hemodialysis for uremia. The mean free IGF-II of these sera was 6.89 ± 0.94(SE) nMol/L. This was significantly greater than free IGF-II in normal sera ($P < 0.01$). The per cent free IGF-II was also elevated, 8.5 ± 0.66 ($P < 0.001$).

We were most interested in measuring free IGF-II in the sera of patients with NICTH because of the markedly reduced fraction of IGF-II present in the 150 kD complexes. In the seven sera studied the free IGF-II was 27.9 ± 3.2 nMol/L (Figure 1). Total IGF-II was measured in four of these sera in the same RIA and the mean was 95 nMol/L.

We next conducted additional studies to determine whether IGFBP-3 or ALS limited the formation of 150 kD complexes in NICTH sera. If ALS was the limiting factor in 150 kD complex formation it should be correctable by the addition of purified ALS to serum. As shown in Figure 2A, 77% of the IGFBP-3 of normal serum was present in fractions 20–24 containing the large complexes and this only increased slightly after the addition of 1.5 μg of purified ALS to 50 μL of serum. When both ALS and 20 ng of IGF-I were incubated with the normal serum the percentage of IGFBP-3 in fractions 20–24 rose to 86.

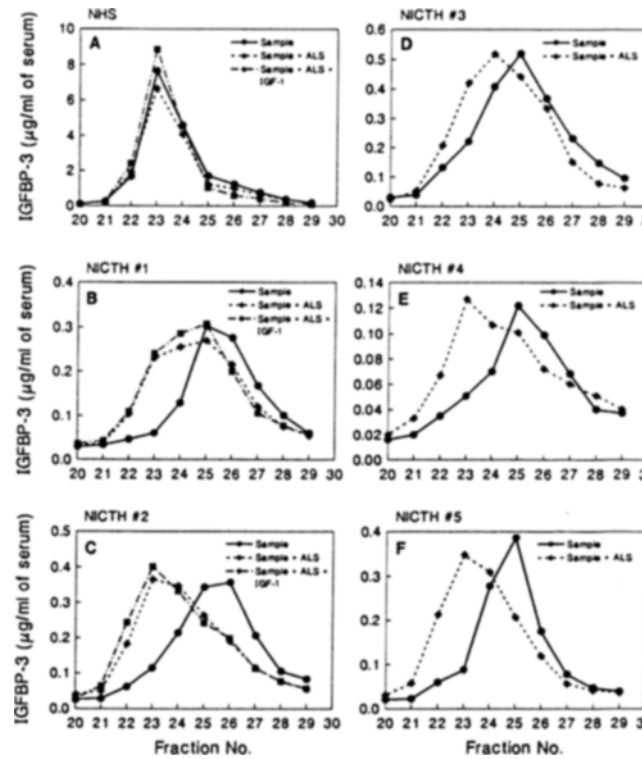

Figure 2 Fifty microliters of normal or NICTH serum, with or without 1.5 μg of purified human ALS and in some cases 20 ng of IGF-I in 50 μl of sodium phosphate buffer, pH 6.5 were incubated for 2 h at 22°C and then passed through a Superose 12 column. Eluent fractions were analysed for IGFBP-3 by RIA

The results obtained with five NICTH sera were different (Figure 2B to E). Before the addition of ALS most of the IGFBP-3 was associated with the smaller BP complexes. After the addition of ALS there was a partial shift of IGFBP-3 into fractions 20 to 24. Even when an excess of IGF-I was added there was not complete restoration of the normal IGFBP-3 distribution. To show this more clearly we have compared the IGFBP-3 in fractions 25–29 (small complexes) in these experiments (Figure 3). In normal serum only about 20% of the IGFBP-3 was present in these fractions but in NICTH sera this was from 60 to 80%. Addition of ALS only went part way in lowering the fraction of IGFBP-3 in the small complexes. Further addition of IGF-I had no additional effect.

Discussion

While it is generally agreed that the concentration of free IGFs is the most important determinant of biological action, measurement of free IGFs has been technically difficult. Separation of free IGFs from bound IGFs by neutral gel filtration (Guler *et al.*, 1987) or ultracentrifugation (Daughaday *et al.*, 1982) failed to separate free IGFs reliably. Hizuka *et al.* (1991) described a neutral Sep-Pak extraction procedure which excludes nearly all the binding protein. Free IGF-I was recovered from the Sep Pak by acid ethanol elution. The free IGF-I of normal sera measured in this way represented about 1.5% of total serum IGF-I. Increased concentrations of free IGF-I were present in the sera of patients with acromegaly and reduced concentrations were present in the sera of patients with hypopituitarism. It is this extraction method which we have used in this paper.

Recently two immunoradiometric assays for free IGF-I have been reported. In the method of Liu *et al.* (1994) serum

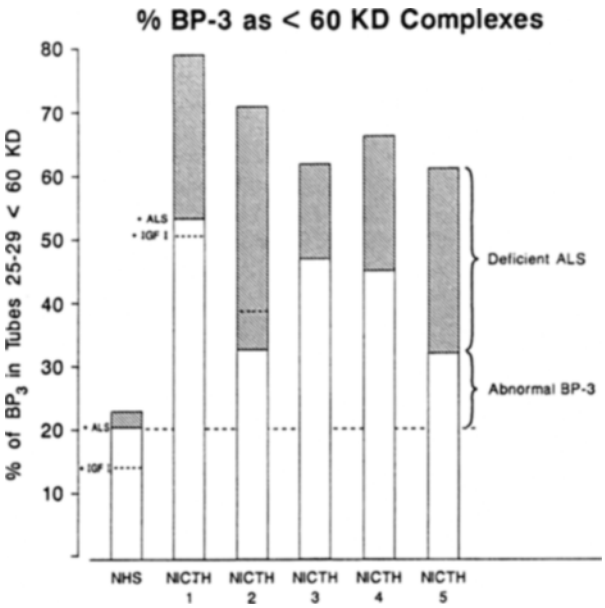

Figure 3 The per cent of total IGFBP-3 present as less than 60 kD complexes (fractions 25 to 29) in normal serum and in five NICTH sera is shown graphicly. The upper lined portion of each column represents the decrease in the small complexes after the addition of ALS. The dashed line across the first three columns shows the results obtained when both ALS and IGF-I were added to the reaction mixture

was incubated with the solid phase first antibody for 2 h before adding the second phase labeled antibody. They reported that 4.4 ± 0.5% of IGF-I of normal serum was free. In the immunoradiometric assay of Takada *et al.* (1994) incubation of the first phase antibody with serum was for less than 10 min before adding the second phase antibody. They reported that free IGF-I of normal adult serum was about 1% of total IGF-I.

Since the completion of the present studies a new method of separation of free from bound IGF-I was reported by Frystyk *et al.* (1994). Native serum was ultrafiltered through Amicon YMT 30 membranes by centrifugation and the concentrations of both free IGF-I and II were measured by ultrasensitive time resolved fluorimmunoassays. The authors reported that free IGF-I was 0.38 ± 0.02% of total IGF-I and free IGF-II was 0.2 ± 0.01% of total IGF-II.

The relative merits of these new methods of measuring free IGFs in serum have not been established. It is possible that the Sep-Paks might steal some IGFs from the most labile of the binding protein complexes, but this should be minimal during the very brief exposure of serum to the Sep-Pak The same criticism would apply to the use of high affinity antibodies in the radioimmunometric method of measurement of free IGF-I. This may explain the higher percentage of free IGF-I observed with a 2 h incubation with the first phase capture antibody (Liu *et al.*, 1994). The ultrafiltraton method of Frystek *et al.* (1994) appears very promising but results may be lowered by very weak and possibly nonspecific binding by serum proteins.

With the Sep-Pak separation method free IGF-II represented about 2% of total IGF-II in normal adults. Despite the partial proteolysis of IGFBP-3 which occurs in pregnancy there is little increase in free IGF-II. This is consistent with the finding that IGFBP-3 in late pregnancy is still capable of forming the 150 kD ternary complex (Suikkari & Baxter, 1992). In uremia there is a decrease in the fraction of IGFs carried in the 150 kD complexes (Goldberg *et al.*, 1982; Lee *et al.*, 1989; Liu *et al.*, 1990). We found that this was associated with a 2- to 8-fold increase in free IGF-II.

Our chief interest in the measurement of free IGF-II was to study sera from patients with NICTH, a condition associated with a marked decrease in the proportion of IGFs carried in 150 kD complexes (Daughaday & Kapedia, 1989; Zapf *et al.*, 1990; Baxter & Daughaday, 1991). As shown in Figure 1, we found that free IGF-II was increased 8–20-fold, consistent with the marked hypoglycemia of these patients and evidence that the increased concentration of IGFBP-2 in this condition has not made IGF-II unavailable to IGFBP-3.

Measurement of free IGF-II should prove to be useful in the diagnosis of NICTH. It is well known that the measurement of total IGF-II is seldom helpful in this condition. Two of us (Daughaday & Trivedi, 1992) found that the concentration of big IGF-II as measured with a RIA directed against the first 21 amino acids of the E domain of proIGF-II is greatly elevated in most cases of NICTH but there are cases where big IGF-II is not increased and processing of proIGF-II to IGF-II is not impaired (Zapf *et al.*, 1992).

With evidence that free IGF-II is readilly available in NICTH sera we turned our attention to the other two reactants in the 150 kD complex, i.e. IGFBP-3 and ALS. Our approach was to add an excess of purifed ALS to serum and observe the change in the distribution of IGFBP-3 between 150 kD and smaller complexes. As shown in Figure 2 addition of ALS to normal serum increased the 150 kD peak only slightly but addition of ALS to NICTH sera increased the fraction of IGFBP-3 in 150 kD complexes but did not restore normal distribution. This indicates that ALS deficiency limits 150 kD complex formation. It also indicates that even when ALS and free IGF-II are present in excess much of the IGFBP-3 of NICTH sera can not enter the ternary complex. This is most likely the result of loss of the ability of the IGF-II:IGFBP-3 binary complex to bind to ALS. Cotterill *et al.* (1991) found evidence of proteolysis of IGFBP-3 and the presence of a serum protease in the serum of one patient with NICTH. This was not found in the three cases studied by Zapf *et al.* (1990) or in the five cases reported by Fukuda *et al.* (1993). It is also possible that some forms of big IGF-II also have impaired ability to participate in ternary complex formation. We conclude that the defect in formation of the 150 kD complex involves both a decrease in available ALS and functional IGF-I:IGFBP-3 binary complex.

Methods

Free IGFs were extracted from serum by a modification of the method of Hizuka *et al.* (1991) developed for IGF-I. Eight C-18 Sep-Paks held in a Waters Sep-Pak Rack were conditioned with 10 ml of methanol and 20 ml of distilled water. Flow rate was then slowed with a screw valve admitting ambiant air connected to the vacuum line. Immediately before extraction 0.2 mL of serum was added to 5 mL of distilled water in a 15 × 150 mm Nunc tube. The contents of the tube were transferred to the Sep-Pak funnel with two 5 mL water washes. Sufficient air was admitted to the vacuum line so that the diluted serum passed through the Sep-Pak in about 5 min. After the diluted serum had passed the Sep-Pak it was followed by 20 mL of water. At this time the lower collection rack was attached which contained 10 × 70 mm Nunc tubes supported in 13 × 100 mm glass tubes containing plugs adjusted so that the lips of the two tubes coincided. Free IGF-II was eluted from the Sep-Paks slowly with 2.5 mL of 70% ethanol, 0.1 M acetic acid. The extract was dried in a Speed Vac and the IGFs were dissolved in 1 mL of RIA buffer (0.03 M Na_2HPO_4, 0.01 M NaEDTA, pH 7.4, 0.02% sodium azide, 0.25% bovine serum albumin (Sigma Chemical)). Total serum IGF-II was extracted by the acid-acetone method of Bowsher *et al.* (1991).

IGF-I was measured by RIA using polyvalent rabbit antibody against IGF-I kindly provided by the National

Pituitary Program. Recombinant human IGF-I (AmGen) was used for iodination and standards. Bound ^{125}I IGF-I was separated from free by the double antibody method. IGF-II was measured with a monoclonal antibody raised against rat IGF-II (Amano Pharmaceutical). Recombinant human IGF-II (Bachem) was used for iodination and standards. Standards made up in RIA assay buffer tend to lose potency even when stored at 4°C. When this was recognized the potency of the working standards were compared to that of freshly prepared standards and appropriate corrrections were made. In our assay recombinant human proIGF-II(E1-21) had a molar potency of 0.5.

The defect in serum binding of NICTH sera was characterized by incubating 50 μL of serum with and without 1.5 μg of purified ALS (Baxter *et al.*, 1989) for 2 h at 22°C in a final volume of 100 μL of buffer (0.05 M sodium phosphate, pH 6.5, 0.1 M sodium chloride, 0.02% sodium azide). In addition two NICTH sera and one normal serum were incubated with both ALS and 20 ng of IGF-I. After incubation the samples were passed through a Superose 12 HR 10/30 FPLC column in the same buffer as previously described (Baxter, 1990). The distribution of IGFBP-3 in each fraction was determined by RIA (Baxter & Martin, 1986).

Sources of sera

Normal sera were obtained from young adult medical students and medical personnel participating in various studies on the Clinical Research Center. Sera from pregnant women at delivery were kindly provided by Dr Hung Winn of the Departent of Obstetrics, Saint Mary's Medical Center, Saint Louis, MO. Sera from uremic patients on chronic hemodialysis were obtained from Dr Edwardo Slatapolsky. Sera from patients with NICTH were provided by Dr L.V. Avioli of Saint Louis (hepatoma), Dr M.A. Emanuele of Chicago (fibrosarcoma), Professor K. Hall of Stockholm (hemangiopericytoma and leiomyosarcoma), Dr R.R. Henry of SanDiego (hemangiopericytoma), Dr P. Rotwein of Saint Louis (prostatic carcinoma), and Dr N.A. Samaan of Houston (adrenal cortical carcinoma). All patients had a history of protracted hypoglycemia requiring large glucose oral intake and in some cases parenteral glucose administration to prevent hypoglycemia. Sera from the United States were shipped frozen on dry ice and stored at −17°C. Sera from Stockholm and sera shipped to Sydney for the study of binding proteins were lyophilized and mailed at ambiant temperature.

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