GROWTH HORMONE-DEPENDENT EXPRESSION AND REGULATION OF INSULIN-LIKE GROWTH FACTOR (IGF) I AND IGF BINDING PROTEIN mRNA LEVELS IN RAT TISSUES IN VIVO

A dissertation submitted to the SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH for the degree of Doctor of Natural Sciences

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Es könnte wohl sein, dass der Wert und die Bedeutung der Naturwissenschaften nicht in erster Linie darin liegt, dass sie uns die moderne Technik und den durch sie ermöglichten Komfort und materiellen Wohlstand brachten, sondern dass ihr eigentlicher, evolutionärer Sinn in der Erweiterung des menschlichen Bewusstseins vom Wunder der Schöpfung besteht.

Albert Hofmann
Einsichten - Ausblicke
TO THOMAS AND MY PARENTS
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SUMMARY

Insulin-like growth factor I (IGF I) plays a major role in regulating growth in vivo and in vitro. Due to its structural similarity to insulin, IGF I exerts classical insulin-like effects on all insulin target tissues. Moreover, IGF I is important for the differentiation of bone, cartilage and muscle. In the circulation, IGF I is found tightly bound to specific binding proteins (IGFBPs). IGFBPs modulate IGF action. Six different forms of IGFBPs have been identified and their cDNAs have been cloned.

The tissue specific expression and regulation of IGF I and IGFBPs were studied in different tissues (liver, kidney, spleen, thymus, brain, muscle, heart, testes and epididymal fat pads = white adipose tissue [WAT]) from hypophysectomized (hypox) rats infused with saline, recombinant human (rh) IGF I or rh growth hormone (GH) in vivo and compared with expression and regulation in untreated normal rats.

The IGF I message is present in all tissues, but it is most abundant in liver and WAT. It is stringently regulated by growth hormone (GH) in liver, muscle and WAT and less in the other tissues. IGF I gene expression is low in WAT from hypox rats and stimulated by GH in vivo and in vitro. However, only in vivo is GH able to raise IGF I tissue levels in WAT. The IGF I mRNA to total RNA ratio is similar in stromal-vascular cells and adipocytes.

IGFBPs are expressed and regulated in a tissue specific manner. IGFBP-2 mRNA is most abundant in testes, IGFBP-3 mRNA in spleen, IGFBP-4 mRNA in liver and IGFBP-5 mRNA in kidney, WAT and skeletal muscle. No GH-dependent regulation of IGFBP expression was observed in kidney, spleen, thymus and testes. In contrast, IGFBPs in liver, WAT, skeletal muscle and heart are regulated by GH. Like GH, IGF I infusion normalized IGFBP-3 and -4 mRNA levels in liver. In contrast to GH, it had no effect on IGFBP-5 mRNA in WAT and
was considerably less effective than GH in raising IGFBP-5 transcript levels in skeletal muscle.

Endogenous IGF I induced by GH-infusion exerts different effects on IGFBP expression than exogenously infused IGF I. These differences may be due to actions of GH at the tissue level including auto/paracrine effects of locally produced IGF I. Locally produced IGF I and IGFBPs in WAT may play a crucial role in the differentiation of adipocytes from stromal-vascular cells.
ZUSAMMENFASSUNG


In Leber, Niere, Milz, Thymus, Hirn, Muskel, Herz, Hoden und epididymalen Fettpolstern (=weisses Fettgewebe WFG) von hypophysektomierten (hypox) Ratten, die mit physiologischer Salzlösung, rekombinan tem humanem (rh) IGF I oder rh Wachstumshormon (GH) infundiert wurden, wurde die gewebe-spezifische Expression und Regulation von IGF I und IGFBPs untersucht und mit der Expression und Regulation in unbehandelten Ratten verglichen.


IGFBPs werden gewebe-spezifisch exprimiert und reguliert. IGFBP-2 mRNA findet man vor allem im Hodengewebe, IGFBP-3 mRNA in der Milz, IGFBP-4 mRNA in der Leber und IGFBP-5 mRNA in Nieren, WFG und Skelettmuskel. In Nieren, Milz, Thymus und Hoden ist die Transkription der IGFBPs unabhängig von GH. Hingegen werden die IGFBPs in Leber, WFG, Skelettmuskel und Herz durch GH reguliert. Wie GH normalisierte auch IGF I die IGFBP-3 und -4 mRNA.
Spiegel in der Leber. Im Gegensatz zu GH jedoch hatte IGF I keine Wirkung auf IGFBP-5 mRNA im WFG und war deutlich weniger wirksam als GH in bezug auf die Stimulierung der IGFBP-5 Transkription im Muskel.

Endogenes IGF I, das durch Infusion von GH induziert wird, hat andere Wirkungen auf die Expression der IGFBPs als exogen infundiertes IGF I. Diese Unterschiede beruhen möglicherweise auf einer GH-Wirkung direkt auf das Gewebe, wobei auto/parakrine Effekte von lokal produziertem IGF I zu berücksichtigen sind. Lokal produziertes IGF I und IGFBPs in WFG spielen vielleicht eine wichtige Rolle bei der Differenzierung von Adipozyten aus Stromazellen.
ABBREVIATIONS

ALS  acid labile subunit
BSA  bovine serum albumin
cDNA complementary desoxyribonucleic acid
DEPC diethyl pyrocarbonate
DNA  desoxyribonucleic acid
EDTA ethylene diamine tetraacetate
GH   growth hormone
HEPES 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
HSA  human serum albumin
IGF  insulin-like growth factor
IGFBP insulin-like growth factor binding protein
kb   kilo bases
kD   kilo dalton
Mr   molecular weight
mRNA messenger ribonucleic acid
MSA multiplication stimulating activity
PBS phosphate buffered saline
PTH  parathyroid hormone
RNA  ribonucleic acid
SDS  sodium dodecylsulfate
Tris 2-amino-2-hydroxymethylpropane-1,3-diol
WAT white adipose tissue
PART I: INTRODUCTION

INSULIN-LIKE GROWTH FACTORS AND THEIR BINDING PROTEINS
1. INSULIN-LIKE GROWTH FACTORS

1.1. History of insulin-like growth factors
Hypophysectomy in rats results in low growth hormone levels and cessation of growth. In 1956, Murphy, Daughaday and Hartnett reported that growth hormone (GH) injection into hypophysectomized (hypox) rats completely restored sulfate incorporation into cartilage (1). For clinical purposes, the authors tried to set up an in vitro GH assay based on $^{35}$S-sulfate incorporation into cartilage. But they found no stimulation of $^{35}$S-sulfate uptake by GH added to the incubation medium. When they compared the sulfation activity of serum from hypox rats in the presence or absence of GH with that of serum from GH-treated hypox rats, only the latter stimulated sulfate uptake. These results prompted Salmon and Daughaday to postulate a hormonally controlled serum activity which they called sulfation factor (2). Sulfation factor also stimulated $^3$H-thymidine incorporation into DNA of cell cultures. Later, this factor was called somatomedin to emphasize its role as a mediator of somatotropin (=GH) action (3).

Serum exerts insulin-like effects on muscle and adipose tissue which are much greater than would be expected on the basis of its insulin content. In 1963, Froesch et al. observed that only a small portion of the insulin-like activity in serum was suppressible with antiserum against insulin (4). Later is was reported that this non-suppressible insulin-like activity (NSILA) consisted of at least two components: 80% was acid-ethanol precipitable NSILA-P and had a molecular weight of about 100'000 and 20% was acid-ethanol soluble NSILA-S with a molecular weight of 7'500 (5).

A third approach to the insulin-like growth factors came from cell biologists. In 1967, Temin reported that the "multiplication stimulating activity" (MSA) of serum measured as stimulation of $^3$H-thymidine incorporation into DNA of
cultured fibroblasts could be replaced by high doses of insulin (6). This factor was partially purified from calf serum and was found to exhibit both multiplication stimulating and nonsuppressible insulin-like activity (7). Isolation and further characterization of MSA were performed using conditioned medium of Buffalo rat liver cells (BRL3A cell line) (8). These cells proliferate in the absence of serum (9). Apparently, they produce MSA as an auto-/paracrine factor to stimulate their own proliferation. However, Nissley et al. demonstrated later by the use of anti-MSA antiserum that proliferation of BRL3A cells is not dependent on MSA (10).

During the purification of sulfation factor it turned out that the sulfation- and DNA-stimulating activities copurified with nonsuppressible insulin-like activity (11,12). In addition, Zingg and Froesch reported that partially purified NSILA stimulated sulfate incorporation into rat and chicken cartilage (13), and Morell and Froesch showed that it stimulated thymidine incorporation into fibroblasts (14,15). These observations led the authors to suggest that NSILA-S and sulfation factor are identical. In 1978, Rinderknecht and Humbel isolated and sequenced two peptides from partially purified NSILA with a molecular weight of about 7'500 (16,17). The sequences of these peptides had about 40% structural homology to proinsulin. Based on this structural homology and on the identical in vitro actions the two peptides were named insulin-like growth factor (IGF) I and II. MSA was isolated from conditioned medium of BRL3A cells by Marquardt et al. and sequenced (18). Except five conservative amino acid substitutions it was identical to human IGF II. Thus, MSA became rat IGF II.
1.2. Physiology of IGFs

The concentrations of IGF I and IGF II in normal adult human serum are about 200 ng/ml (26 nM) and 700 ng/ml (93 nM), respectively. The main production site of circulating IGFs is the liver (19, 20, 21). But IGFs are also produced by other tissues (22). More than 95% of the circulating IGFs are bound to specific binding proteins and do not exert insulin-like effects. The concentrations of free IGF I and IGF II range between 1 and 2 nM.

The most prominent biological effects of the IGFs are those which have led to their discovery: stimulation of $^{35}$S-sulfate and $^3$H-thymidine incorporation into cartilage (2), insulin-like effects on muscle and adipose tissue (4) and stimulation of cell proliferation (7). Today, a large spectrum of IGF effects is known. Below, their metabolic and growth-promoting effects and their role in cell differentiation are described.

1.2.1. Metabolic effects

In vitro: IGFs elicit metabolic effects in vitro within seconds to minutes (short-term effects). They stimulate glucose uptake, glycogen and lipid synthesis and inhibit lipolysis in adipose tissue (23) and they enhance glucose uptake, glycogen synthesis and glycolysis in muscle (24). On a molar basis, the potency of IGFs in muscle is 10 to 20 times and in adipose tissue 50 to 100 times lower than that of insulin.

In vivo: Bolus injections of recombinant human (rh) IGF I into rats (25) or human volunteers (26) cause hypoglycemia and lower serum free fatty acid levels. When administered by subcutaneous infusion during six days into normal human subjects IGF I causes a variety of hormonal and metabolic effects: It lowers insulin, C-peptide, GH and IGF II levels as well as triglyceride
concentrations and the total to high density lipoprotein (HDL) cholesterol ratio. High concentrations of IGF I decrease also blood glucose levels (27).

1.2.2. Growth promoting effects

In vitro: IGF I and IGF II stimulate DNA, RNA and protein synthesis in a variety of cells in vitro and lead to cell proliferation (28). In contrast to metabolic effects, the mitogenic actions of IGF are long-term and need hours to days before they are detectable.

In vivo: In 1981, Schoenle et al. infused IGF I for six days into hypox rats via subcutaneously implanted osmotic mini-pumps (29). Due to the slow, continuous infusion, the animals did not become hypoglycemic. Body weight and tibial epiphyseal width increased. Similar results were obtained with rh IGF I in genetically GH-deficient Snell dwarf mice (30) and in hypox rats (31). These experiments also showed that IGF I administration increased the weight of kidneys, spleen and thymus. In 1986, Scheiwiller et al. infused streptozotocin-diabetic rats with rh IGF I (32). The animals grew, although hyperglycemia and glucosuria continued.

1.2.3. Effects on differentiation

Evidence is accumulating that IGFs support differentiation of cells of mesodermal origin. For example, IGF I enhances alkaline phosphatase activity, an indicator of osteoblast differentiation in osteoblast-like cells prepared from newborn rats, whereas cell number increases only slightly (33). IGF I also plays an important role in muscle differentiation. It enhances the formation of myotubes from chick embryonic cells (34) and stimulates terminal myogenic differentiation by induction of the myogenin gene expression (35). IGF I also
participates in differentiation of adipocytes from preadipocytes (36) and in red blood cell differentiation (37). In vivo, it stimulates erythropoiesis in hypox rats (38).

1.3. IGF Receptors

The two types of biological activities of IGFs and insulin, acute metabolic and long-term mitogenic effects, have been explained by the existence of two different receptors. Evidence for separate membrane receptors was first reported by Megyesi et al. (39). They performed binding studies on lymphocytes, adipocytes and fibroblasts and showed that the insulin displacing potency of NSILA-S correlated with its insulin-like bioactivity. In addition, two types of IGF receptors have been discovered (40).

1.3.1. The type 1 IGF receptor and the insulin receptor

The structures of the type 1 IGF receptor and the insulin receptor are similar (Figure 1). They are both composed of two \( \alpha \)-subunits of 135 kD and two \( \beta \)-subunits of 90 kD linked by disulfide bonds (40). The \( \alpha \)-subunits represent the major part of the extracellular domain and include the ligand binding site. The \( \beta \) chains consist of an extracellular, a transmembrane and an intracellular domain.

Binding of the ligand activates a tyrosine kinase located on the intracellular domain of the \( \beta \)-subunit. Receptor autophosphorylation is followed by phosphorylation and activation of intracellular proteins like IRS-1 (41) and MAP-2 kinase (42). The homology between the type 1 IGF and the insulin receptor is most pronounced in the tyrosine kinase domain (84% sequence identity is found).
Heterodimer formation ($\alpha\beta$) is sufficient for ligand binding, but autophosphorylation and activation of the intrinsic tyrosine kinase is dependent on formation of the heterotetramer ($\alpha_2\beta_2$) linked by disulfides. In the insulin receptor, phosphorylation of the receptor $\beta$-subunit appears to be catalyzed by the kinase of the neighbouring $\beta$-subunit of the disulfide-linked receptor complex (43).

Since IGFs and insulin as well as type 1 IGF and insulin receptors are structurally related, it is not surprising that they can crossreact with each other's receptor. IGF I binds to the type 1 receptor with an affinity of $1.5 \times 10^{-9}$ M (44). The affinity of the type 1 IGF receptor is 3 and 100 times lower for IGF II and insulin, respectively. The binding affinities of the insulin receptor are: insulin $\gg$ IGF II $>$ IGF I.

Although it has been proposed initially that acute metabolic effects of insulin and IGFs are mediated by insulin receptors and growth effects by type 1 IGF receptors, it is now clear that this is an oversimplification and that many exceptions to this rule are known. For example IGFs appear to stimulate glucose transport and metabolism via type 1 IGF receptors in mouse muscle (24, 45) and human fibroblasts (46).

These data indicate that there are no inherent differences in the abilities of the two receptor kinases to mediate various responses and suggest that the different physiological roles of these two peptides are determined by the distribution of the two receptors on different cells and/or the pharmacodynamics of the two peptides.
1.3.2. The type 2 IGF receptor

Most cells express a second, type 2 IGF receptor which exhibits a higher affinity for IGF II than for IGF I. Insulin does not bind to this receptor. The type 2 IGF receptor is a single chain polypeptide of 250'000 kD containing a short intracellular domain, a transmembrane domain and a large extracellular domain consisting of 15 repetitive sequences (Figure 1, 47). It is identical to the cation-independent mannose-6-phosphate (Man-6-P) receptor. The binding sites for
IGF II and Man-6-P are on different domains so that both ligands can bind simultaneously. This duality of the type 2/Man-6-P receptor complicates speculations about its physiological relevance. Furthermore, the necessity of IGF binding to this receptor is questionable since Man-6-P receptors from chicken and *xenopus laevis* have no binding sites for IGF II. Apparently, IGF II acts via the IGF type 1 receptor in these animals. The biological function of the type 2 IGF receptor is not yet known.

### 1.4. Structure of the IGFs

#### 1.4.1. Primary structure

The primary structures of IGFs from at least nine different species are known (Figure 2). In all of them, IGF I consists of 70, IGF II of 67 amino acid residues. They make up 4 different domains: A, B (similar to insulin), C (corresponding to the connecting peptide [C-peptide] of proinsulin) and D (not present in insulin). Analogous to (pro)insulin, the molecule is stabilized by three intrachain disulfide bridges. The A- and B-chains of insulin are 41% and 43% identical to the A- and B-domains of IGF I and II, respectively (17). No homology is found between the C-peptide of proinsulin and the C-domain of IGFs. The sequence identity between IGF I and II is 62%.

Larger forms of IGF I and IGF II with carboxy terminal extensions have been characterized (48). The levels of these variants in normal adult serum are low (big-IGF II) or undetectable (big IGF I). However, in patients with extrapancreatic tumor hypoglycemia up to 80% of IGF II circulates as big IGF II (49, 50). Sera of children and adults with chronic renal failure contain big pro-IGF IA presumably because of defective clearance of this precursor (51).
INSULIN-LIKE GROWTH FACTORS

IGF I

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>GPETLCGAELVDALQFVCCGDRGFYPNKPT</td>
</tr>
<tr>
<td>Bovine</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Porcine</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Ovine</td>
<td>----------------------------------------</td>
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<td>Rat</td>
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<td>Mouse</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Chicken</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Xenopus</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Hagfish</td>
<td>LS----S----T----DT----F--VPQH</td>
</tr>
</tbody>
</table>

**Figure 2**: Amino acid sequences of IGF I and II. Amino residues are given in single-letter code. Dashes signify identical to the human sequence. The peptide domains are indicated in the bottom line. (Sequences of human, bovine, porcine, ovine, rat and mouse IGF I and II are from [28], chicken [52], xenopus [53], hagfish [54], amphioxus [55])

1.4.2. Gene structure

IGF I gene and mRNA

The human and rat IGF I genes contain at least 5 exons distributed over 90 kb (Figure 3). Exons 1 and 2 encode portions of the signal peptide, exons 2 and 3 encode the four domains of the mature peptide, exon 3 the proximal portion of the E-domain and exons 4 and 5 contain the E-regions of preproIGF-IB and IGF-IA respectively as well as 3' untranslated sequences (56, 57). Two types of human mRNAs are generated by alternative splicing using either exon 5 (IGF-IA) or exon 4 (IGF-IB). Two distinct polyadenylation sites in exon 5 lead to
the synthesis of two IGF-IA mRNAs which differ in size. In the rat, processing of the primary gene transcript is more complex than in the human. Additional alternative splicing of the RNA precursor at the 5' end as well as use of at least 5 different polyadenylation sites generate mRNAs with different sizes.

Northern blot analysis of human and rat IGF I mRNA reveals a series of bands ranging in size between 0.7 to more than 7.0 kb. The differently sized mRNAs are generated by mechanisms like alternative splicing and variable polyadenylation. The sequence of the 7.0 kb mRNA species differs from the smaller forms in the length of the 3' untranslated sequence.

* Figure 3: Structure of the human IGF I gene and mRNA species. The double blocks for exons 1B and 1C represent the two alternative transcription initiation sites. The black blocks in each exon show the regions coding for the IGF I precursor. The mature polypeptide is encoded by exons 2 and 3. Shaded boxes represent untranslated sequences. The asterisks indicate different polyadenylation sites. (from [59])
IGF II gene and mRNA

The human IGF II gene (Figure 4) consists of 9 exons and contains 4 promoters (59). The mature IGF II molecule is encoded by exons 7, 8 and part of exon 9. The use of the different promoters and the two polyadenylation sites in exon 9 lead to the transcription of five different mRNA species. Interestingly, the promoters are expressed in a tissue- and development-specific manner: P2, P3 and P4 are active in fetal tissues, whereas P1 is used in adult liver. A fetal type of promoter use is observed in different tumors expressing high levels of IGF II (49, 60, 61). A small 1.8 kb cleavage product can be detected when hybridizing with a 3'-specific probe.

Figure 4: Structure of the human IGF II gene and mRNA species. The promoters are indicated by arrows (P1-4). The asterisks show two alternative polyadenylation sites. The mature IGF II polypeptide is encoded by exons 7, 8 and part of exon 9. (from [59])
1.5. Regulation of IGFs

In the last three decades, numerous papers have confirmed the GH-dependence of IGF I discovered by Daughaday in 1957 including clinical studies on acromegalic and hypopituitary patients (28). Serum IGF I levels in patients with GH deficiency (pituitary dwarfism) and absolute GH-resistance (Laron-type dwarfism) are about 5% and 15% of normal (48). Although it is well established that GH regulates IGF I at the level of gene expression, no putative GH response element has been found in the IGF I gene promoter (62). Beside GH, hepatic IGF is regulated by insulin (63, 64).

Furthermore, nutrition influences IGF I levels. In man and rat, IGF I serum levels decline during fasting and are raised by feeding. Resistance to the growth promoting effects of GH caused by nutrient restriction is probably due to a hepatic block of IGF I synthesis.

Finally, serum IGF I in vivo is dependent on the developmental state (28). At birth the concentration of IGF I in cord blood is about one third of normal adult serum. During childhood, IGF I levels rise constantly and reach adult levels just before puberty. During puberty, there is a 2- to 3-fold rise of circulating IGF-I in girls and boys. This rise has been proposed to be due to an increase of the amplitude of the pulsatile GH secretion and to the increase of sex steroids. Adult serum concentrations remain constant until the age of 60. Thereafter, they decline.

GH regulates IGF I production in many tissues. GH secretion itself is, among other factors, regulated by IGF I acting as a feedback inhibitor directly on the pituitary gland. Furthermore, GH stimulates somatostatin production of the hypothalamus (65). It is not clear, whether this GH-effect is direct or mediated by IGF I.

In contrast to IGF I, IGF II levels are less stringently regulated by GH. IGF II may contribute to GH-independent growth because the serum concentrations of
IGF II of GH-resistant and GH-deficient children remain about 40% and 30% of normal, respectively (48). Hence, IGF II is not a classical somatomedin. There are no significant changes of IGF II serum concentrations during puberty.
2. IGF BINDING PROTEINS

2.1. Historical Background
Evidence for the existence of IGF binding proteins was reported by Zapf et al. in 1975 (66). In an IGF radioreceptor assay using fibroblast extract as matrix and charcoal for separation of bound and free $^{125}$I-labeled IGF, addition of increasing amounts of serum resulted in increased binding instead of displacement. These results indicated that serum bound IGF tracer. In subsequent studies, the specificity of this binding was demonstrated. Neither insulin nor GH were able to displace IGF tracer. Radiolabeled IGF preparations, preincubated with human serum and chromatographed on Sephadex G-200 at neutral pH, bound specifically to two protein pools which appeared as a 150 kD and a 50 kD peak. Similar observations in human and rat serum were made by other groups (67, 68). In addition, Moses and coworkers showed that the 150 kD complex was GH-dependent, whereas the small 50 kD complex appeared to be inversely regulated by the GH-status. When $^{125}$I-IGF was injected into rats in vivo, the majority of protein-bound radioactivity in serum was initially associated with the 50 kD pool, but between 20 min and 4 h most of it shifted to the 150 kD pool (69).

2.2. The family of IGF binding proteins
Two methods have been used for the detection of IGFBPs: gel filtration and ligand blotting. Prior to gel filtration, the sample was preincubated with radiolabeled IGF tracer. The radio chromatogram obtained after chromatography on Sephadex G-200 at neutral pH provided information about the size distribution pattern of exchangeable IGF binding sites.
Figure 5: Upper panel. Sera from hypox rats treated with saline (——), rhGH (200 mU/d for 8 days, - - -), or rhIGF I (200 µg/d for 8 days, — — —) were incubated with 125I-IGF II for 24 h at 4°C and fractionated on a Sephadex G-200 column at neutral pH. The radiochromatographic pattern represents free binding capacity and exchangeable binding capacity of the sera. Lower panel. Fractions obtained after gel chromatography on Sephadex G-200 were pooled as indicated (pool I, II and III) and analyzed by ligand blotting (methodical details are given in the Material and Methods section page 36). The IGFBP-3 triplet in pool I of normal rat serum (NRS) is absent in the corresponding pool of hypox serum (HYP), but reappears in pool I of sera from GH-treated hypox rats. IGF I infusion also induces the IGFBP-3 triplet, but it circulates at a lower molecular form in these rats. This result indicates a direct regulation of the acid labile subunit by GH (see part IGFBP-3, page 17). The 32 kD binding protein detected in pool I of serum from normal and GH-treated hypox rats is truncated IGFBP-3. The 32 kD band in pool III of serum from hypox rats is IGFBP-2. The 24 kD band in pool III from normal and hypox rats represents the deglycosylated form of IGFBP-4. (from [70])
In 1986, Hossenlopp et al. used a modification of the classic Western blot for the detection of IGFBPs. After separation on a non-reducing polyacrylamide gel and transfer to a membrane, the samples were incubated with radiolabeled IGF instead of using anti-serum for the detection (more detailed in Material and Methods, page 36) (71). Specifically bound IGF was then visualized by autoradiography. This method (called ligand blotting) provided information about the composition of binding activity found in serum. In normal adult human serum, a double band at apparent molecular masses of 42 and 45 kD, a band around 36 kD and one at 24 kD were found. When the peaks obtained by gel filtration were ligand blotted, the double band around 45 kD and a 32 kD band eluted in the high molecular weight complex (150 kD) and the 36 kD and 24 kD bands in the low molecular form (50 kD). Later, when the proteins had been isolated and sequenced, the two bands at 42 and 45 kD were identified as glycosylation variants of the same protein. It was called IGFBP-3. The 36 kD form was designated IGFBP-2 and the 24 kD protein IGFBP-4. The 32 kD band eluting with the 150 kD complex turned out to be truncated IGFBP-3. The IGFBP pattern of normal adult rat serum is shown in Figure 5. In contrast to human, the rat IGFBP-3 is detected as a triplet band on ligand blots.

Six different IGFBPs have been isolated and cloned. Since 70 - 80% of the total serum IGF is bound to the 150 kD complex containing exclusively IGFBP-3, this BP will be considered first.

2.2.1. IGFBP-3

IGFBP-3 has been purified from human plasma in 1986 (72). Based on N-terminal and internal amino acid sequence data, the human IGFBP-3 cDNA has been cloned from a human liver cDNA library (73). Later, rat IGFBP-3 has been isolated from rat serum and cloned (74, 75). The human and the rat
proteins are 264 and 265 amino acids long (M_r=28'700, protein core only) (Table 1). Human IGFBP-3 has 3 and the rat protein has 4 potential N-glycosylation sites. The human IGFBP-3 is 83% homologous to rat IGFBP-3. A single copy gene is located on chromosome 7 in the human genome (76). It spans 8.9 kb and contains five exons. The IGFBP-3 mRNA is 2.4 kb long (73).

In contrast to all other IGFBPs, IGFBP-3 after binding IGF combines with an acid-labile subunit (ALS) to form the 150 kD complex found in adult human and rat serum (77). ALS is a 84-86 kD glycosylated protein with a protein core of 70 kD (78). It has no intrinsic IGF-binding activity. Hence, preformation of a binary complex of IGF*IGFBP-3 is required before the ternary complex can form. Studies with structural analogs of rhIGF I showed that the interaction of ALS with IGF*IGFBP-3 in the ternary complex is dependent on structural determinants on IGF I distal to the IGFBP-3 binding domain, although it does not bind IGF I itself (79). ALS is synthesized in vitro by hepatocytes (80). Recently, ALS has been cloned from a human liver cDNA library (81). The mature protein consists of 578 amino acids and contains 18-20 leucin-rich repeats of 24 amino acids. These repeats are also found in a number of other proteins that participate in protein-protein interactions.

IGFBP-3 is the predominant BP found in adult serum (approximately 100 nM [82]). However, it also occurs in other body fluids. Low concentrations of IGFBP-3 have been detected in human and rat lymph (83, 84), in human and rat colostrum (85, 86) and rat milk as well as in human follicular fluid (87).

GH is an important regulator of circulating IGFBP-3 concentrations (73). IGFBP-3 is also induced by IGF I infusion in hypox rats (70). However, the induced IGFBP-3 is not associated with the 150 kD complex (see Figure 5). Only after GH-administration is the induced IGFBP-3 found in the 150 kD complex. These results indicate that ALS is dependent on GH.
IGFBP-3 is synthesized and secreted in many cell lines and cell types. Since IGFBP-3 is the predominant BP found in serum, the liver was believed to be the main site of its production. However, primary cultures of hepatocytes do not produce IGFBP-3 (88). Therefore, the production of IGFBP-3 in liver may be restricted to non-parenchymal cells like endothelial cells.

Vascular endothelial cells (89), neonatal and adult fibroblasts (90, 91), chondroblasts and osteoblasts produce IGFBP-3. The regulation of IGFBP-3 in these cells varies greatly with the cell type. IGF I stimulates IGFBP-3 expression in cultured bovine, but not in human fibroblasts (92). In neonatal human fibroblasts, transforming growth factor-β (TGFβ) is a potent stimulator of IGFBP-3 production (93). Production of IGFBP-3 in rat osteoblasts is stimulated by GH, estradiol (E2) and IGF I (94, 95). IGFBP-3 expression is completely suppressed by cortisol treatment both in human fibroblasts and rat osteoblasts. Prostaglandin E2 and parathyroid hormone stimulate IGFBP-3 production in bone cells but not in fibroblasts (96, 97). This stimulatory effect is blocked by TGFβ. E2 and epidermal growth factor (EGF) stimulate IGFBP-3 production by porcine granulosa cells of medium-sized follicles. But follicle stimulating hormone (FSH) and TGFβ both suppress basal and E2/EGF stimulated IGFBP-3 production in these cells (98).

2.2.2. IGFBP-1

IGFBP-1 has been cloned from cDNA libraries of decidua (99, 100, 101) and HepG2 hepatoma cells (102). The cDNA codes for a 259 amino acid residue preprotein with a putative signal sequence of 24 residues. The mature protein consists of 234 amino acids and has a molecular mass of 25 kD (M_r=25'300) (Table 1). It contains a PEST region (Pro, Glu, Ser, Thr-cluster) which is found in proteins with short half-lives. The sequence also includes a Arg-Gly-Asp
(RGD) tripeptide which may serve as a cell attachment site (103). The human IGFBP-1 gene contains four protein-coding exons and is located on chromosome 7 (99). IGFBP-1 mRNA is 1.6 kb in length (101). IGFBP-1 is expressed in the secretory endometrium and decidua, and is abundant in human amniotic fluid. In adult plasma, IGFBP-1 concentrations are low (about 2 nM [82]), but they increase during fasting, diabetes and GH-deficiency indicating an inverse regulation by insulin. In vitro studies employing cultured HepG2 cells showed that insulin decreased IGFBP-1 synthesis and secretion independent of the glucose concentration in the medium (104). Recently, nuclear run-on assays performed with hepatic nuclei from normal, diabetic and insulin-treated diabetic rats demonstrated that insulin decreases transcription of IGFBP-1 to normal control levels already 1 h after injection. Normalization did not require restoration of euglycemia (105). Dexamethasone increases IGFBP-1 secretion and hepatic mRNA expression in rats and in H4-II-E rat hepatoma cells in vitro (106, 107).

2.2.3. IGFBP-2
Rat IGFBP-2 has been cloned from a BRL-3A cDNA library by the groups of Rechler and Schwander (108, 109). Using the rat cDNA, human IGFBP-2 was cloned from a fetal human liver cDNA library (110). Like IGFBP-1, rat and human IGFBP-2 contain an RGD sequence, but no potential N-glycosylation site. Although human IGFBP-2 is > 85% homologous with rat IGFBP-2, it is 19 amino acids longer (289 amino acids as compared to 270 amino acids, \( M_r = 31'125 \) and 29'500, respectively [Table 1]). The human IGFBP-2 gene has been localized to chromosome 2. The rat and human IGFBP-2 genes comprise 4 exons, each of which contains coding sequences (111, 112, 113). The human gene spans 32 kb due to a 27 kb intron. The human and rat promoter
regions lack a TATA or CAAT consensus sequence but have a high GC content which is often associated with housekeeping genes. In human and rat, a single transcript of 1.5 and 1.6 kb was found (110, 109).

IGFBP-2 is the predominant BP in the rat fetus. It is abundant on day 21 of gestation in rat liver, stomach, brain, kidney and lung (114). After birth, mRNA levels initially rise (day 1) and then fall to 10% of fetal levels by day 21 after birth. In the adult rat, IGFBP-2 is absent in liver, muscle and heart, but expressed in kidney, brain and testes (109).

Hypophysectomy induces hepatic IGFBP-2 expression. But in contrast to IGF I mRNA levels, GH-infusion in hypox rats is not able to normalize hepatic IGFBP-2 expression. Fasting increases IGFBP-2 production in the rat (115).

By comparing gene transcription and mRNA levels Tseng and coworkers recently showed that the regulation of the hepatic IGFBP-2 by fasting and refeeding occurs at the transcriptional level (116). High levels of IGFBP-2 mRNA are also found in livers from diabetic rats (117). In contrast to IGF I-treatment, insulin therapy lowers IGFBP-2. In cultured rat hepatocytes, insulin is the main regulator of IGFBP-2 expression, whereas GH does not change IGFBP-2 mRNA (118).

IGFBP-2 is most abundant in fetal rat and human serum. In human adult serum levels of IGFBP-2 are between 200 and 500 ng/ml (7-17 nM [82]). IGFBP-2 is also present in human and rat cerebrospinal fluid (119, 120) and amniotic fluid (84). Many cell types express IGFBP-2. As is true for IGFBP-3, the regulation of IGFBP-2 depends on the cell type. For example, retinoic acid induces IGFBP-2 mRNA in rat hepatocytes, but suppresses it in rat osteoblasts (121).

The suppressive effect of insulin on IGFBP-2 mRNA observed in hepatocytes is not detected in osteoblasts. In contrast to IGFBP-3, IGFBP-2 expression is not changed by GH or PTH. Triiodothyronine (T3) stimulates IGFBP-2 mRNA transcription and IGFBP-2 secretion in rat osteoblasts (122).
2.2.4. IGFBP-4

IGFBP-4, originally called inhibitory IGFBP due to its inhibitory effect on bone cell proliferation, was purified from human TE-89 osteosarcoma cell-conditioned medium (123), adult human (124) and rat serum (125) and human prostatic cell-conditioned medium (126). cDNAs coding for IGFBP-4 were isolated from cDNA libraries of rat liver and human placenta (127), human TE-89 osteosarcoma cells (128) and human osteosarcoma cells (124). The mature form of rat IGFBP-4 consists of 233 amino acids (Mr = 26'000), whereas the human homolog is four amino acids longer (Table 1). In contrast to all other IGFBPs, it contains two extra cysteine residues, i.e. 20 cysteines. It occurs in an N-glycosylated (residue 125) and nonglycosylated form (124, 129). The human IGFBP-4 gene is located on chromosome 17 (131). The human and rat IGFBP-4 transcripts are 2.3 and 2.6 kb long, respectively (124, 128).

Northern blot analysis in rat tissues revealed that the gene transcription in liver is highly active. In all other tissues, there were hardly any transcripts detectable (127). IGFBP-4 mRNA has also been identified in human bone cells, embryonic skin fibroblasts and TE-89 osteosarcoma cells (128). Little is known about the regulation of IGFBP-4. In vitro studies of human osteoblast-like cells suggest that PTH stimulates the expression of IGFBP-4 and that the PTH-effect is mediated through a cAMP-dependent mechanism (128). In addition, 1,25-dihydroxyvitamin D₃, another major calcium regulating hormone, stimulates IGFBP-4 both at the protein and mRNA level in normal human bone cells and human osteosarcoma cells (130).
2.2.5. IGFBP-5

IGFBP-5 was isolated from adult rat serum (131), human U-2 osteosarcoma cell-conditioned medium (132) and human bone extracts (133). The IGFBP-5 cDNA clone was found by screening a rat ovary cDNA library with primers based on the N-terminal sequence of the isolated protein (131). Another approach for the cloning of IGFBP-5 was made by Kiefer et al. (134). They used two primers designed towards two conserved regions in the COOH-terminal third of the known IGFBPs to perform polymerase chain reaction (PCR) with osteosarcoma cDNA as a template. The PCR products were then used to screen an osteosarcoma cDNA library and full length cDNA clones were isolated. The mature protein derived from human cDNA contains 252 amino acids ($M_r$=28'550). The human IGFBP-5 gene is located on chromosome 5 (131). High expression of a 6.0 kb IGFBP-5 mRNA was found in kidney, although the mRNA was also detected in other tissues like lung, heart, brain and stomach (131). The regulation of IGFBP-5 mRNA is not known yet.

2.2.6. IGFBP-6

IGFBP-6 was purified from human (124) and rat serum (135) and porcine follicular fluid (135), and cDNA clones were isolated from osteosarcoma, rat ovary and human placenta cDNA libraries (124, 135). The mature human protein is 237 amino acids long ($M_r$=25'980) and contains a potential N-glycosylation site which is not used (Table 1). IGFBP-6 lacks two (human) or four (rat) of the N-terminal cysteines found in other IGFBPs. The human IGFBP-6 gene is located on chromosome 12 (131). According to Shimasaki et al. IGFBP-6 is expressed in lung, testis, large and small intestine, adrenal, kidney, stomach, spleen, heart, brain and liver of adult male rats (135).
Table 1

Molecular characteristics of the human IGFBP proteins, the sizes of their mRNAs and the chromosomal location of their genes are summarized.

<table>
<thead>
<tr>
<th></th>
<th>Mr (kD)</th>
<th>amino acids</th>
<th>cysteine residues</th>
<th>glycosylation type</th>
<th>transcript size</th>
<th>chromosomal location</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-1</td>
<td>25'300</td>
<td>234</td>
<td>18</td>
<td></td>
<td>1.6 kb</td>
<td>7</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>31'325</td>
<td>289</td>
<td>18</td>
<td></td>
<td>1.5 kb</td>
<td>2</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>28'700</td>
<td>264</td>
<td>18</td>
<td>N-</td>
<td>2.4 kb</td>
<td>7</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>25'980</td>
<td>237</td>
<td>20</td>
<td>N-</td>
<td>2.3 kb</td>
<td>17</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>28'550</td>
<td>252</td>
<td>18</td>
<td></td>
<td>6.0 kb</td>
<td>5</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>25'980</td>
<td>237</td>
<td>16</td>
<td>O-</td>
<td>1.3 kb</td>
<td>12</td>
</tr>
</tbody>
</table>

2.3. Comparative structural aspects of the IGFBPs

A comparison of the amino acid sequences of the six human IGFBPs is shown in Figure 6. Homology is restricted to the N-terminal and the C-terminal thirds, whereas the middle thirds do not display homology. All six IGFBPs contain between 16 (IGFBP-6) and 20 (IGFBP-4) cysteine residues at identical positions. Twelve cysteines are clustered near the amino terminus and six are near the carboxyl terminus.

Determination of free sulfhydryl groups by carboxymethylation of rhIGFBP-3 revealed that most likely all cysteine residues are involved in disulfides (136). The exact disulfide structures of the molecules are not yet known, but the number and specific alignment of these cysteines imply an important role for the correct folding of the molecules and their IGF binding activities.
IGFBP-3, -4 and -6 contain Asn-linked glycosylation sites which are only occupied in IGFBP-3 and -4. Human IGFBP-6 purified from cerebrospinal fluid is O-linked but not N-glycosylated. Glycosylation does not affect the affinity of IGFBP-3 and -6 for IGF I and II nor does it alter the preferential affinity of IGFBP-6 for IGF II (137, 138).
Table 2

<table>
<thead>
<tr>
<th>IGFBPs</th>
<th>125I-IGF I</th>
<th>125I-IGF II</th>
</tr>
</thead>
<tbody>
<tr>
<td>n BP-1a</td>
<td>1.6 x 10^9</td>
<td>1.8 x 10^9</td>
</tr>
<tr>
<td>II</td>
<td>5 x 10^8</td>
<td>2 x 10^9</td>
</tr>
<tr>
<td>rh BP-2b</td>
<td>7 x 10^9</td>
<td>2 x 10^9</td>
</tr>
<tr>
<td>n BP-2</td>
<td>1.2 x 10^9</td>
<td>2.2 x 10^9</td>
</tr>
<tr>
<td>rh BP-3</td>
<td>1 x 10^10</td>
<td>1 x 10^10</td>
</tr>
<tr>
<td>n BP-3</td>
<td>2 x 10^10d</td>
<td>3 x 10^10d</td>
</tr>
<tr>
<td>rh BP-4</td>
<td>2 x 10^10</td>
<td>1.9 x 10^10</td>
</tr>
<tr>
<td>rh BP-5</td>
<td>2.6 x 10^10</td>
<td>1.1 x 10^10</td>
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<td>8.5 x 10^8</td>
<td>1.9 x 10^9</td>
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<tr>
<td>n BP-6</td>
<td>7.2 x 10^8</td>
<td>1.2 x 10^9</td>
</tr>
<tr>
<td>2-3 x 10^9a</td>
<td>3 x 10^9a</td>
<td></td>
</tr>
</tbody>
</table>

IGFBP-1 and -2 contain an RGD sequence which may enhance their ability to bind to cells. IGFBP-1 exists in several phosphorylated and nonphosphorylated forms in cell cultures (141) and in human fetal serum and amniotic fluid (142). Preferred phosphorylation sites are Ser 101 and Ser 169, and less Ser 119. Phosphorylation enhances the affinity of IGFBP-1 for IGF I (141).

All members of the IGFBP family bind IGF I and IGF II. IGFBP-1,-3,-4 and -5 bind both IGFs with about equal affinity (K_a 0.6 - 2.0 x 10^10 M^-1), whereas IGFBP-2 and -6 have a lower affinity for IGF I than for IGF II (Table 2). IGFBP-6 binds IGF II 20 times better than IGF I (139).
The structural homology of the IGFBPs suggests that they share a common
IGF binding domain. Little is known about the IGF binding regions on IGFBP
molecules. Mutants of IGFBP-1 with a deletion of the last 20 C-terminal amino
acids completely lost the IGF binding ability as determined by ligand blotting
(143). Point mutations in the C-terminal region of IGFBP-1 did not cause
alterations in the IGF binding properties except when Cys-226 was changed
into a Tyr which resulted in dimer formation and loss of IGF binding. Deletion of
the 60 N-terminal residues resulted in loss of IGF binding (144). However,
most point mutations introduced in the N-terminal region did not affect IGF
binding except for a Cys-38 to Tyr mutation which completely abolished it.

More data are available about regions on the IGF molecule involved in the IGF-
IGFBP binding. Early studies showed that a truncated form of IGF I lacking the
first three N-terminal amino acids (des1-3 IGF I) bound very poorly to bovine
IGFBP-2 (=MDCK-BP) (145). Experiments with mutated IGF analogs revealed
that the residues 4, 5, 15 and 16 of the B-domain and amino acids 49-51 of the
A-domain are required for binding to IGFBP-1 and -2 (146). Another group
compared tyrosine labelling of free and IGFBP-2-bound IGF and demonstrated
that Tyr-60 and -59 labelling was reduced in bound IGF I and II respectively
(147). Hence, these tyrosine residues directly participate in the binding or are
protected by IGFBP-2 binding.

2.4.  Physiological aspects

2.4.1. Serum pool of IGF

In normal serum, more than 90% of IGF I and more than 98% of IGF II are
bound to IGFBPs. 70 - 80% of total IGF (IGF I and II) is associated with a 150
kD complex consisting of IGF, IGFBP-3 and ALS (148). Its bioavailability is
largely restricted by the capillary barrier (83). Thereby, the circulating 150 kD complex in serum serves as an IGF reservoir. 20 - 30% of total serum IGF is associated with IGFBPs in a 50 kD complex. This complex crosses the capillary barrier, and its IGF may therefore become available to tissue IGF receptors.

2.4.2. Protection against hypoglycemia
The total circulating IGF concentration is approximately 100 nM compared to a fasting insulin concentration of about 100 pM. Although IGF is 50 to 100 times less potent than insulin with respect to its insulin-like actions, these concentrations of circulating IGF would cause constant hypoglycemia unless most of the serum IGF were bound to specific binding proteins.

2.4.3. Prolongation of serum half-life
In 1976, Cohen and Nissley observed that the serum half-life of IGF was GH-dependent (149). In vivo studies with iv injections of $^{125}$I-IGF tracer into healthy adults revealed that free IGF disappears from the circulation with a half-life of 10 min (150). Low molecular weight complexed IGF (50 kD pool) decreased with a half-life of 20-30 min. In contrast, IGF levels in the high molecular weight complex (150 kD pool) were relatively constant over 6 h and disappeared from the circulation with a half-life of 12-16 h.

2.4.4. Dual role of IGFBPs on IGF action
Originally, IGFBPs were found to inhibit most IGF actions, but recently, evidence for an inhibitory and stimulating role of IGFBPs is accumulating. A Cys-281 variant of rhIGFBP-2 inhibited proliferation and collagen synthesis of
fetal rat calvaria (151). IGF I-stimulated DNA and glycogen synthesis in human osteosarcoma B-10 cells is completely inhibited by rh IGFBP-3, -4 and -5 at a 10 to 100 fold molar excess (152, 139). rhIGFBP-6 only blocked IGF II-induced $^3$H-thymidine incorporation, consistent with its 20 fold higher affinity for IGF II. A stimulatory effect of IGFBP-1 has been described by Elgin et al. (153). They found a 4-fold stimulation of $^3$H-thymidine incorporation in various cell types. A dual role for IGFBP-3 in IGF-stimulated DNA synthesis was reported for human skin fibroblasts (154). Co-incubation of IGFBP-3 and IGF I resulted in a decrease of DNA synthesis, whereas pre-incubation for 8 to 24 h with IGFBP-3 potentiated IGF I-stimulated $^3$H-thymidine incorporation. It is not clear, whether this effect is due to decreased down-regulation of the type 1 IGF receptor during the pre-incubation or to sensitization of the cells towards IGF I. Blocking of IGF I-induced receptor down-regulation by purified bovine IGFBP-3 has been observed in cultured bovine fibroblasts (155). Stimulation of IGF I-induced DNA synthesis has also been described for IGFBP-5 (156, 133). Membrane association of IGFBP-3 may be followed by proteolytic cleavage resulting in decreased affinity of the IGFBP and release of cell-bound IGF I. This may provide a mechanism explaining the potentiating activity of IGFBP-3 in pre-incubation experiments (157). Membrane associated IGFBP-3 is also present on human skin fibroblasts and is released in response to IGF I (158). Proteolytic degradation of IGFBP-3 was first described in term pregnancy serum (159, 160, 161). Later, protease activity was demonstrated in seminal plasma, serum from critically ill patients and conditioned media from several cancer cell lines (162, 163). The proteolytic activity of seminal plasma could be ascribed to the prostate-specific antigen (PSA). The IGFBP-3 protease from pregnancy serum was further characterized as a cation-dependent tryptic-like serine protease (164). Recently, in human fibroblasts a protease specific for IGFBP-4 was found (165). Interestingly, this protease was strictly dependent
on IGFs for activation. However, the mechanism for this remains to be determined.

IGFBPs modulate IGF actions at the cellular level. Whether they act in an inhibitory or in a stimulating manner depends on the cell type, the assay conditions (pre- versus co-incubation), on the molar ratio between IGF and IGFBPs, on their dissociation constants and on the presence of proteases or other mechanisms.
PART II: EXPERIMENTAL WORK

A. TISSUE SPECIFIC EXPRESSION AND REGULATION OF IGF I AND IGFBPs IN THE RAT
32 INTRODUCTION

1. INTRODUCTION

The original somatomedin hypothesis of Daughaday and coworkers stated that GH does not act directly on peripheral tissues but rather through a mediator (somatomedin) produced under the influence of GH (3). Later, this hypothesis of indirect GH action was challenged by Isakson and collaborators (166). They injected GH directly into one of the proximal tibial epiphyseal growth plates of hypox rats and observed increased bone growth only on the injected side. This observation questioned the role of circulating IGF I in GH action. Subsequently, it was shown that co-infusion of anti IGF I antiserum together with GH into the arterial supply of one hindlimb of hypox rats abolished the growth effect of GH completely (167). These results led to the concept that GH stimulates bone growth by inducing local production of IGF I, which in turn stimulates growth in an auto-/paracrine fashion. Further experiments demonstrated that GH increases the number of IGF I-immunoreactive cells in the proliferative zone of the growth plate of hypox rats as well as local IGF I mRNA expression (168, 169).

The fact that IGF I gene expression and protein synthesis are found in many tissues (170, 22) supports the now well established concept that IGF I does not act exclusively in an endocrine but also in an auto-/paracrine manner. However, the relative contributions of systemic IGF, locally produced IGF and directly acting GH are still under dispute. Sara and Hall suggested that a truncated form of IGF I (des1-3-IGF I) acted in an auto-/paracrine and intact IGF I in an endocrine manner (171). Later it was reported that exon 1B IGF I transcripts are expressed mainly in liver, whereas exon 1C transcripts are found in all tissues, including liver (172). These two classes of IGF I mRNAs lead to preproIGF1s which differ in the N-terminal part of the signal sequence. LeRoith and Roberts suggested that preproIGF I transcribed from exon 1C may be cleaved by signal peptidase to generate a proIGF I molecule lacking the N-
terminal tripeptide of the B-domain which exhibits decreased binding affinity for IGFBPs (173). The other form of preproIGF I would be appropriately processed to generate intact IGF I which is secreted into the circulation and exerts systemic actions.

Since IGF actions are modified by IGFBPs, local production of IGFBPs could make a major contribution to the auto-/paracrine concept of IGF action. Locally produced IGFBPs may retain IGF at the site of synthesis or adsorb IGF from the circulation and thereby provide a local reservoir of IGF which is less susceptible to degradation than uncomplexed IGF.
1.1. **Aims of this study**

Despite many papers published on the regulation of IGFBPs, in vivo and in vitro studies are limited to the regulation of IGFBP-1, -2 and -3 in serum, liver and kidney. Therefore, the aim of this study was to examine the tissue-specific expression and regulation of IGF I and IGFBPs in vivo. A hypox rat model was used. Hypox rats were infused with saline, IGF I and GH. Normal rats served as physiological controls. The comparison between IGF I- and GH- infusion was of special interest: It provided the possibility to evaluate systemic (IGF I-infusion) versus systemic plus auto-/paracrine (GH-infusion) actions of IGF I, although IGF-independent effects of GH in the latter model could not be excluded.

In particular, the following questions were addressed:

1) What is the expression pattern of IGF I and IGFBPs in different tissues of normal and hypox rats?

2) How are IGF I and IGFBPs regulated by IGF I or GH administered to hypox rats in vivo?

3) Can infused IGF I mediate the actions of infused GH on IGF I and IGFBP expression in different tissues? This would have to be assumed on the basis of the somatomedin hypothesis.
2. MATERIAL AND METHODS

2.1. Animals

Hypophysectomy was carried out in 7 week-old male Tif RAI rats (courtesy of Dr. K. Müller and M. Cortesi, CIBA-Geigy, Basel). They were kept at 25°C on a 12-h light/dark cycle and had free access to food and water. Animals who gained less than 2 g/week during 5 weeks were selected for infusion. Alzet mini osmotic pumps (model 2001, Alza, Palo Alto CA, USA) were filled with solvent (0.1 M HOAc/0.9 % NaCl), recombinant human (rh) IGF I (provided by Dr. K. Müller, CIBA-Geigy AG, Basel) in 0.1 M HOAc/0.9 % NaCl or rhGH (Novo Nordisk, Gentofte, Denmark) in H2O. The minipumps were implanted under the subcutis of the abdomen under ether anesthesia. Groups of five animals were infused for 6 days with solvent, rhIGF I at a rate of 300 μg/rat/day or rhGH at a rate of 200 mU (67 μg)/rat/day. Healthy age-matched control animals were kept under identical conditions, but received no further treatment.

Food and water intake as well as body weight were measured daily at 0830 during the infusion period. Two days after the implantation of the minipumps, calcein (2 mg in 0.5 ml of 2% NaHCO₃ [Fluka, Buchs]) was injected i.p. After six days of infusion, the rats were anesthetized with Innovar Vet (Pitman Moore, Washington Crossing, NJ), 0.2 ml/100g body weight, and bled by aortic puncture. Blood glucose was determined immediately using a Beckman glucose analyzer. Ten organs, i.e. liver, kidney, spleen, thymus, brain, heart, soleus and gastrocnemius muscle, testes and epididymal fat pads were excised, blotted on a filter paper and weighed. They were immediately frozen in liquid nitrogen and stored at -80°C until RNA was isolated. Blood was kept on ice for 30 min and centrifuged for 15 min at 1'000 x g and 4°C. Serum was stored in 1 ml samples at -20°C for further analysis.
2.2. Biochemical methods

2.2.1. Insulin and IGF I determination

Serum insulin was measured with a rat insulin radioimmunoassay (RIA) kit (Novo Nordisk, Gentofte, Denmark).

Endogenous and infused IGF I were separated from serum binding proteins by chromatography on Sep Pak C18 cartridges (Waters, Millipore, Milford MA, USA) according to the protocol supplied by Immunonuclear (Stillwater MN, USA). After reconstitution with 1 ml of phosphate-buffered saline/0.2 % HSA, pH 7.4, samples were assayed at two or three different dilutions. Immunoreactive IGF I was determined by RIA (174) using a rabbit anti human IGF I antiserum at a final dilution of 1:10'000 and rat IGF I (gift from Dr. M. Kobayashi, Fujisawa, Japan) or rhIGF I as a standard.

2.2.2. Analysis of IGFBPs by ligand blotting

The method described in detail by Hossenlopp et al. (71) was used with slight modifications (70). 3 μl of serum was mixed with 20 μl of 0.1 M Tris-HCl buffer pH 6.8, containing 4% SDS, 0.02 M NaEDTA and 24% (vol/vol) glycerol and electrophoresed for 5 h at 170 mV on SDS/15% polyacrylamide slab gels under nonreducing conditions (except the 14C-labeled molecular weight marker: Rainbow Marker, Amersham, UK). The proteins were transferred on nitrocellulose by electroblotting for 2 h at 0.6-1.0 A under cooling with running tap water using a Transblot cell (Bio-Rad, Richmond CA, USA).

Ligand blots were processed as described (70): The air-dried nitrocellulose sheets were soaked for 30 min at 4°C in "saline" (0.15 M NaCl containing 0.01 M Tris-HCl, pH 7.4, NaN₃ at 0.5 g/l, and 3% Nonidet P-40 [Sigma, St. Louis MO, USA]), incubated overnight at 4°C in "saline"/1% human serum albumin (Fluka), and finally for 10 min in "saline"/0.1% Tween 20 (Serva,
Heidelberg, Germany). Each sheet was then incubated at room temperature for 6 h in a sealed plastic bag with 18 ml of "saline" containing 1% human serum albumin/0.1% Tween 20 and 4 x 10^6 cpm of 125I-labeled IGF II (Anawa, Wangen/Zürich). After two washings for 15 min in "saline", the membranes were air-dried and exposed for 1-3 days at -70°C to an X-ray film (Kodak, X-Omat AR) in a Kodak X-Omatic cassette.

2.3. Molecular biological methods

2.3.1. Chemicals

The following chemicals used for RNA isolation were from Gibco BRL (Basel), ultra pure grade: caesium chloride, guanidine isothiocyanate and agarose. SDS, Tris, EDTA, sodium citrate, diethyl pyrocarbonate (DEPC) and 3-[N-morpholino]propane-sulfonic acid (MOPS) were from Sigma. Competent cells were from Gibco BRL (Cat. No. 8263SA). Restriction enzymes used for the preparation of cDNAs were from Boehringer Mannheim (Rotkreuz). All solutions used for RNA isolation were made up with DEPC-treated H2O (except Tris stock solutions) and sterilized by autoclaving. All glassware used for RNA isolation buffers was "baked" for 4 h at 140°C to destroy RNase activity.

2.3.2. RNA isolation

Total RNA was isolated according to standard procedures (175). 0.5 to 1 g of frozen tissue was homogenized in a Polytron homogenizer at 4°C in 3 ml of ice-cold 4 M guanidine isothiocyanate containing 5 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol and 0.5% sarcosine. The homogenates were centrifuged at 500 x g for 5 min at 4°C to reduce foaming. 1 g of cesium
chloride was dissolved in each 2.5 ml of homogenate. This solution was layered onto a 1.2 ml cushion of 5.7 M cesium chloride in 0.1 M EDTA (pH 7.5) in a 4.2 ml polyallomer tube (Kontron, Zürich). RNA was separated from proteins and DNA by centrifugation at 35,000 rpm and 18°C for 16 h in a TST60.4 swing-out rotor (Kontron). The supernatant was removed with a Pasteur pipette down to 5 mm above the bottom of the tube. The walls were dried with Kleenex tissues before the remaining fluid was discarded. The inside walls of the tubes were cleaned a second time before the transparent RNA pellet was dissolved in 100 µl of 10 mM Tris-HCl, pH 7.4, 5 mM EDTA and 1% SDS and transferred to a fresh tube. The centrifugation tube was washed with another 100 µl aliquot and both aqueous phases were combined. They were extracted with 200 µl of a 4:1 mixture of chloroform and 1-butanol. 190 µl of the aqueous upper phase were transferred to a fresh tube and the remaining organic phase was reextracted with 190 µl of 10 mM Tris-HCl, pH 7.4, 5 mM EDTA and 1% SDS. The RNA was finally precipitated with 40 µl of 3 M NaOAc, pH 5.2, and 2 volumes of absolute ethanol. After 2 h at -20°C, the RNA was recovered by centrifugation in an Eppendorf centrifuge at 15,000 rpm and 4°C for 20 min and washed with ice-cold 70% ethanol in DEPC-treated H2O. RNA was stored in 70% ethanol at -70°C. The RNA pellets were dried in a Speed Vac (Savant, Farmingdale NY, USA) and then dissolved in DEPC-treated H2O. Concentrations were determined spectrophotometrically (1 OD_{260nm}=40 mg/ml RNA). Integrity of the isolated RNA was checked on a 1% agarose gel stained with ethidium bromide.

2.3.3. cDNAs

Human IGFBP-3 cDNA was cloned by Dr. J. Zapf from a HepG-2 cDNA library at Chiron Corporation (same nucleotide sequence as in [73]). Human IGFBP-4
to -6 cDNA probes were provided by Dr. M. Kiefer (Chiron Corporation, CA, USA) ([124, 134] In the original publication IGFBP-4 had been tentatively designated as IGFBP-5, IGFBP-5 as IGFBP-6 and IGFBP-6 as IGFBP-4). The rat IGF I cDNA corresponds to the genomic sequences between nucleotide 2054 of exon 1 and nucleotide 868 of exon 5 (57). Rat IGF I cDNA, rat IGFBP-2 cDNA (109) and rat IGFBP-3 cDNA were a gift from Dr. J. Schwander (Kantonsspital Basel). β-actin cDNA was from Dr. C. Schmid and yeast 18S cDNA was provided by Dr. M. Kalousek (Universitätsspital Zürich). All cDNAs were obtained as plasmids or plasmid containing bacteria.

2.3.4. Transformation of competent cells

Aliquots of competent cells were thawed on ice. 50 μl of cells were mixed with 10 ng of plasmid in a maximal volume of 5 μl and incubated for 30 min on ice. The cells were heat-shocked for 45 sec in a 42°C waterbath and placed on ice for another 2 min. Then, 950 μl of room temperature Luria Broth (LB: 10 g bacto-tryptone, 5 g bacto-yeast extract and 10 g NaCl per litre, adjusted with 5 N NaOH to pH 7.0) was added and the mixture was transferred to a Falcon 2059 tube. After 1 h of shaking at 180 rpm at 37°C, 100 μl of bacteria were plated on LB/ampicillin (amp, 50 μg/ml) agar plates and grown overnight at 37°C.

2.3.5. Preparation of plasmid DNA

5 ml of LB/amp were inoculated with a colony from the agar plate and grown overnight at 37°C under shaking at 180 rpm. The next day, 150 ml of LB/amp were inoculated with 1 ml of the bacteria culture and grown overnight. The plasmid DNA was isolated using the Plasmid Midi Kit (Qiagen, distributed by
Kontron). In brief, the bacteria were centrifuged for 15 min at 10,000 x g and the pellet was dissolved in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA containing 100 µg/ml RNase A. The cells were lysed by the addition of 200 mM NaOH, 1% SDS, and the lysate was neutralized with 3.0 M KOAc, pH 5.5. Separation of precipitated proteins, chromosomal DNA and cell debris was achieved by centrifugation at 12,000 x g for 30 min at 4°C. The plasmid DNA was finally purified on the Qiagen column and recovered by precipitation with isopropanol. The plasmid concentration was determined spectrophotometrically (1 OD_{260nm} = 50 mg/ml DNA).

2.3.6. Purification of cDNA

1 µg of plasmid DNA was digested with the appropriate restriction enzyme(s) (5-10 units) in a volume of 20 µl and the digest was analyzed on a 1% agarose gel. When digestion was complete and showed the expected insert size, a 50 µg digestion was performed in a volume of 1 ml. The digestion reaction was precipitated by the addition of 0.1 volume 3 M NaOAc, pH 5.2, and 2 volumes of ethanol. The digestion products were collected by centrifugation, dissolved in 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA) and separated on a 1% agarose gel. The gel was stained with ethidium bromide (5µg/ml) and the insert band was excised under UV light with a razor blade. The DNA was purified from the agarose gel using the Sephaglas Band Prep Kit (Pharmacia, Dübendorf). The cDNA concentration was determined by comparison with different marker concentrations on a 1% agarose gel.
2.3.7. Northern Blotting

Denatured RNA (20 µg) was electrophoresed on a 1% agarose gel containing 2 M formaldehyde, transferred onto a nylon membrane (Hybond-N, Amersham, UK), and RNA was fixed by UV-cross-linking. The filters were prehybridized at 42°C for 2 h in a solution containing 50% formamide, 5 x Denhardt's (0.02% [w/v] Ficoll™, 0.02% [w/v] polyvinyl pyrolidone), 5 x SSPE (20x = 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA pH 7.7), 0.2% SDS and 100 µg/ml of heat-denatured salmon sperm DNA. The cDNA probes were labeled by random primer extension using a commercial kit (Boehringer Mannheim, Rotkreuz) and [α-32P] deoxycytidine 5'-triphosphate (3000 Ci/mmol, Amersham, UK). The radiolabeled cDNA probes had specific activities of 2-4 x 10^9 cpm/µg DNA. Hybridization was performed in the same solution as described above with 10-15 ng (about 2 x 10^7 cpm) of labeled cDNA per filter. After 48 h of incubation at 42°C, the filters were washed twice for 15 min at room temperature in 2 x SSPE, 0.1% SDS and subsequently 3 times in 0.1 x SSC, 0.1% SDS for 20 min at 55°C (β-actin, rIGF I, rIGFBP-2 and -3), at 65°C (18S rRNA probe) or 3 times in 0.7 x SSPE, 0.1% SDS for 20 min at 54°C (hIGFBP-3, -4 and -5 probes).

2.4. Other methods

2.4.1 Determination of tibial epipysial width

The tibia test was performed according to Greenspan et. al. (176). Tibiae were split longitudinally using a razor blade, kept in 10% formaldehyde adjusted to pH 7-7.4 with NaOH and washed under running tap water for 1 h. After 15 min in acetone, they were watered for another 30 min. Staining was performed by a 90 sec incubation in a freshly prepared 2% silver nitrate solution followed by a 2 min exposure under a halogen lamp. Tibiae were photographed under a
stereo-microscope (Wild, Heerbrugg) at a 25-fold magnification using Polaroid film type 667. Ten different sections of the tibial epiphysial width were measured on the photograph and the mean value was calculated.

2.4.2. Determination of longitudinal bone growth
Calcein labeled tibia were kept for 2-3 days in 40 % ethanol. Dehydration of tibia was performed by a sequential rise of the ethanol concentration: 24 h in 70 %, 24 h in 80 %, 24 h in 90 %, 24 h in 96 % and 3 x 24 h in 100 % ethanol. After 4 x 12 h in xylol, the tibiae were embedded in a methacrylic acid methyl ester polymer as follows: 4 x 12 h in MMA I, 2 days in MMA II and finally polymerization in MMA III for at least 14 days. Polymerization was performed in tightly closed polyethylenvials in a waterbath at 30°C in an oven. The water level was always above the acrylate level so that the heat produced by the polymerization reaction could be rapidly diverted.

MMA I 100 ml methacrylic acid methyl ester (MMA, Fluka No. 64200)
MMA II 100 ml MMA + 2g di-benzoylperoxid (Fluka No. 33581)
MMA III 100 ml MMA + 4g di-benzoylperoxid + 25 ml plastoid N
(Bender and Hobenheim, Zurich)

100 - 150 µm thin sections of the fixed tibiae were photographed under a fluorescence microscope (Dialux 20, Leitz) at different magnifications. Longitudinal bone growth was determined by measuring newly synthesized bone (distance between the fluorescent zone and the border of the epiphysis) at ten different sites and calculating the mean value.
3. RESULTS

3.1 Growth parameters

Growth parameters of hypox rats infused with saline, IGF I or GH for 6 days were compared with those of normal age-matched rats. They are summarized in Table 3.

Saline-infused hypox animals did not gain weight during six days of infusion, whereas body weight increased 14.6 and 18.8 g/6d, respectively, in IGF I- and GH-infused animals as compared to 24.6 g/6 days in age-matched controls. Food intake was higher in the IGF I- and GH-treated groups than in hypox controls.

Tibial epiphysial width increased 144% in IGF I-treated animals and 232% in GH-treated rats as compared to hypox controls. Treatment with IGF I and GH led to a 290% and 540% stimulation of longitudinal bone growth, respectively (Figure 7).

Table 3

Growth parameters of normal, hypox, IGF I-treated and GH-treated hypox rats. (mean values ± SD, n=5).

<table>
<thead>
<tr>
<th>treatment</th>
<th>body weight gain (g/6d)</th>
<th>epiphysial width (μm)</th>
<th>longitudinal bone growth (μm/day)</th>
<th>food intake (g/day/rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>24.6 ± 5.0</td>
<td>508 ± 51</td>
<td>1135 ± 36.5</td>
<td>18.8 ± 0.50</td>
</tr>
<tr>
<td>hypox</td>
<td>-1.6 ± 2.3</td>
<td>161 ± 9</td>
<td>38.9 ± 8.5</td>
<td>4.74 ± 0.19</td>
</tr>
<tr>
<td>hypox + IGF I</td>
<td>14.6 ± 2.0</td>
<td>231 ± 19</td>
<td>153 ± 35.7</td>
<td>6.20 ± 0.34</td>
</tr>
<tr>
<td>hypox + GH</td>
<td>18.8 ± 3.1</td>
<td>373 ± 32</td>
<td>250 ± 33.2</td>
<td>6.70 ± 0.10</td>
</tr>
</tbody>
</table>
Figure 7: Sections of calcein-labeled tibiae at different magnifications are shown (A: 4.3-fold; B: 16.5-fold; C, E and G: 6.6-fold; D, F and H: 26.2-fold). The calcein labeled zone is indicated by an arrow. The white bar in B, D, F and H represents 500 μm. The substance between the calcein labeled zone and the border of the epiphysis represents newly synthesized bone. (A) and (B) normal control rats. (C) and (D) hypox rats. (E) and (F) IGF I-treated hypox rats. (G) and (H) GH-treated hypox rats.
Body and organ weights are shown in Table 4. In contrast to GH, IGF I-treatment of hypox rats resulted in significantly increased kidney and spleen as well as decreased liver weights. Thymus mean values of IGF I- and GH-infused hypox rats were also elevated when compared to normal and hypox rats, but the inter-individual variations of thymus weights were considerable. In contrast to GH, IGF I-infusion lowered epididymal fat weight in hypox rats.

Table 4

<table>
<thead>
<tr>
<th>organs</th>
<th>normal</th>
<th>hypox</th>
<th>hypox + IGF</th>
<th>hypox + GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>body weight</td>
<td>404 ± 24</td>
<td>140 ± 7</td>
<td>149 ± 8</td>
<td>163 ± 6</td>
</tr>
<tr>
<td>liver</td>
<td>15.94 ± 2.36</td>
<td>4.630 ± 0.341</td>
<td>4.247 ± 0.385</td>
<td>5.976 ± 0.502</td>
</tr>
<tr>
<td>kidney</td>
<td>2.322 ± 0.149</td>
<td>0.685 ± 0.055</td>
<td>0.953 ± 0.062</td>
<td>0.904 ± 0.05</td>
</tr>
<tr>
<td>spleen</td>
<td>0.717 ± 0.088</td>
<td>0.220 ± 0.027</td>
<td>0.452 ± 0.067</td>
<td>0.351 ± 0.035</td>
</tr>
<tr>
<td>thymus</td>
<td>0.822 ± 0.141</td>
<td>0.251 ± 0.109</td>
<td>0.402 ± 0.099</td>
<td>0.434 ± 0.085</td>
</tr>
<tr>
<td>heart</td>
<td>1.132 ± 0.072</td>
<td>0.377 ± 0.026</td>
<td>0.456 ± 0.055</td>
<td>0.459 ± 0.01</td>
</tr>
<tr>
<td>testes</td>
<td>3.637 ± 0.275</td>
<td>0.292 ± 0.023</td>
<td>0.330 ± 0.084</td>
<td>0.273 ± 0.04</td>
</tr>
<tr>
<td>brain</td>
<td>2.040 ± 0.123</td>
<td>1.786 ± 0.095</td>
<td>1.775 ± 0.091</td>
<td>1.900 ± 0.077</td>
</tr>
<tr>
<td>soleus</td>
<td>0.335 ± 0.030</td>
<td>0.124 ± 0.013</td>
<td>0.122 ± 0.010</td>
<td>0.150 ± 0.011</td>
</tr>
<tr>
<td>gastrocnemius</td>
<td>4.721 ± 0.323</td>
<td>1.797 ± 0.168</td>
<td>1.937 ± 0.150</td>
<td>2.119 ± 0.09</td>
</tr>
<tr>
<td>epididymal fat</td>
<td>4.111 ± 1.117</td>
<td>0.684 ± 0.108</td>
<td>0.450 ± 0.104</td>
<td>0.743 ± 0.20</td>
</tr>
</tbody>
</table>
3.2 Serum parameters
The metabolic and hormonal parameters of the four experimental groups are compiled in Table 5. Blood glucose levels were 11.06 mM in normal and 8.32 mM in hypox rats. GH-infusion had no effect on the blood glucose level, whereas IGF I-infusion exhibited a tendency to lower glucose levels. However, this IGF I-effect was not significant.

Hypophysectomy caused a decrease of IGF I serum levels from 1595 ng/ml in normal age-matched control animals to 53.6 ng/ml. IGF I levels in rats infused with rh IGF I were 332.6 human ng equivalents/ml. Since human IGF I has a four-fold higher affinity to the antiserum used than rat IGF I, the IGF I levels measured after rh IGF I-infusion are comparable to the IGF I levels in normal rats. GH-infusion elevated IGF I serum levels to 774.6 ng/ml.

Hypophysectomy caused a decline of the serum insulin levels from 1.9 ng/ml in normal rats to 0.184 ng/ml. GH-infusion slightly increased serum insulin, whereas IGF I-treatment tended to lower insulin levels, although this decrease was not significant.

<table>
<thead>
<tr>
<th>treatment</th>
<th>glucose (mM)</th>
<th>IGF I (ng/ml)</th>
<th>insulin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>11.06 ± 2.23</td>
<td>1595 ± 190</td>
<td>1.896 ± 0.965</td>
</tr>
<tr>
<td>hypox</td>
<td>8.32 ± 0.98</td>
<td>54 ± 7</td>
<td>0.184 ± 0.057</td>
</tr>
<tr>
<td>hypox + IGF I</td>
<td>6.80 ± 1.55</td>
<td>333 ± 55</td>
<td>0.156 ± 0.023</td>
</tr>
<tr>
<td>hypox + GH</td>
<td>8.48 ± 0.81</td>
<td>775 ± 71</td>
<td>0.360 ± 0.100</td>
</tr>
</tbody>
</table>
3.3 Serum IGF binding protein patterns

The serum binding protein patterns of the four experimental groups were analyzed by ligand blotting. As shown in Figure 8, the IGFBP-3 triplet around 45 kD was decreased in hypox rat serum, but restored by IGF I- and GH-treatment. A double band at 32/33 kD, probably IGFBP-2, was slightly increased by IGF I-treatment. GH-treatment "induced" a broader band of similar size, which probably represents truncated IGFBP-3.

![Figure 8: Ligand blot of sera from the four experimental groups. NRS, normal rat serum; HYPOX, serum from hypox rats. Molecular weight markers are indicated (M_r x 10^{-3}).]
3.4. Tissue specific expression of IGF I and IGFBP mRNAs

3.4.1. IGF I mRNA

IGF I and IGFBP mRNA expression was studied in nine different organs from normal rats, i.e. liver, kidney, spleen, thymus, brain, heart, muscle (gastrocnemius), testes and white adipose tissue (WAT). As shown in Figure 9, IGF I mRNA was expressed at high levels in liver and WAT. There was also significant IGF I expression in spleen and testes. Only very low levels were detected in kidney, thymus, brain, heart and skeletal muscle.

Figure 9: Tissue specific expression of IGF I mRNA. 20 µg of total RNA extracted from normal rat tissue was separated on a 1% agarose gel, blotted on a nylon membrane and hybridized with a rat IGF I cDNA probe.
3.4.2. IGFBP mRNAs

The tissue distribution of IGFBP mRNAs is shown in Figure 10. IGFBP-2 mRNA was detected as a single band of 1.7 kb. It was expressed in normal testes, brain and WAT. The message for IGFBP-3 of 2.5 kb was detected in liver, kidney, spleen, heart, testes and WAT. Very low levels were found in skeletal muscle. The 2.2 kb transcript of IGFBP-4 mRNA was most abundant in liver and at much lower levels in WAT. IGFBP-5 mRNA was the largest BP transcript with a size of 6.5 kb. Its expression was found in kidney, brain, heart, skeletal muscle and WAT.

**Figure 10**: Tissue specific expression of IGFBP mRNAs.

The same blot shown in Figure 9 was stripped and rehybridized with cDNA probes specific for IGFBP-2, -3, -4 and -5.
3.5. Tissue specific regulation of IGF I and IGFBP mRNAs

3.5.1. Liver

Liver is the major source of circulating IGF I (21). It was therefore not surprising to find high IGF I mRNA levels in this organ. As shown in Figure 11 and Table 6, IGF I mRNA decreased after hypophysectomy to 12.5% of normal and was restored to 69% of normal with GH-infusion. IGF I-infusion suppressed its own expression in liver down to 2%. IGFBP-2 transcripts were absent in normal liver, but expression was found after hypophysectomy. IGF I treatment of hypophysectomy animals induced a further increase of IGFBP-2 mRNA levels, an effect not observed with GH-infusion (Table 6). IGFBP-3 expression in liver was low, but significant. It disappeared after hypophysectomy and reappeared during IGF I- and GH-infusion. The third BP species found in liver was IGFBP-4. Its expression was not significantly influenced by hypophysectomy or by IGF I- or GH-infusion.

3.5.2. Muscle

IGF I expression in skeletal muscle was low (see also Figure 9). The decline of IGF I mRNA after hypophysectomy was more pronounced in gastrocnemius than in soleus muscle (17 and 44% of normal, respectively; Figure 12 and Tables 7 and 8). In contrast to gastrocnemius, where IGF I-infusion only partly restored normal levels, there was a complete normalization of IGF I expression by IGF I-treatment in soleus muscle. However, GH-infusion resulted in a 1- to 3-fold elevation above normal of IGF I mRNA levels in both gastrocnemius and soleus muscle. The expression of IGFBP-5 mRNA correlated with IGF I transcript levels. In soleus muscle, there was only a small decrease after hypophysectomy. IGF I stimulated IGFBP-5 mRNA abundance 2-fold above normal in soleus, but only 42 ± 9 % in gastrocnemius muscle. GH-infusion caused a 4-fold stimulation above normal in soleus and gastrocnemius muscle.
Figure 11: IGF I and IGFBP mRNA expression in rat liver. 20 μg of total liver RNA from normal (1), hypox (2), IGF I-treated (3) and GH-treated hypox rats (4) was analyzed by Northern blotting.
IGF I and IGFBP-2, -3 and -4 mRNA levels detected in total liver RNA from 3 to 5 different rats were analyzed by video-densitometry. All values were normalized against the corresponding values obtained for the 18S ribosomal RNA. IGF I, IGFBP-3 and -4 mRNA levels of normal and IGFBP-2 mRNA levels of hypox rats were taken as 100% as indicated by an asterisk. Data are given as percentage.

<table>
<thead>
<tr>
<th></th>
<th>normal</th>
<th>hypox</th>
<th>hypox + IGF I</th>
<th>hypox + GH</th>
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</thead>
<tbody>
<tr>
<td>IGF I</td>
<td>100*</td>
<td>8.8</td>
<td>1.2</td>
<td>40</td>
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<td></td>
<td>100*</td>
<td>9.1</td>
<td>3.4</td>
<td>44</td>
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<td>2.9</td>
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<td></td>
<td>100*</td>
<td>8.3</td>
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<tr>
<td></td>
<td>100</td>
<td>12.5 ±5.2</td>
<td>2.2 ±1.2</td>
<td>69 ±27</td>
</tr>
<tr>
<td>IGFBP-2</td>
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<td>100*</td>
<td>296</td>
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<td></td>
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<td>100</td>
<td>327 ± 88</td>
<td>106 ± 57</td>
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<td>100*</td>
<td>29</td>
<td>84</td>
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<tr>
<td></td>
<td>100</td>
<td>60 ±21</td>
<td>86 ±52</td>
<td>83 ±31</td>
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Figure 12: IGF I and IGFBP-5 mRNA expression in soleus and gastrocnemius. 20 μg of total RNA from normal (1), hypox (2), IGF I-treated (3) and GH-treated hypox rats (4) was subjected to Northern blotting.
Table 7
IGF I and IGFBP-5 mRNA levels in soleus muscle from 3 experiments were measured by video-densitometry and corrected with β-actin values. As indicated by an asterisk, IGF I and IGFBP-5 mRNA levels from normal control rats were taken as 100%. Data are expressed as percentage of normal.

<table>
<thead>
<tr>
<th></th>
<th>normal</th>
<th>hypox</th>
<th>hypox + IGF I</th>
<th>hypox + GH</th>
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<td></td>
<td>100</td>
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<td>111 ± 50</td>
<td>359 ± 162</td>
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<td>IGFBP-5</td>
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<td>70</td>
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<td>369</td>
</tr>
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</table>

Table 8
IGF I and IGFBP-5 mRNA levels in gastrocnemius muscle from 4 experiments were measured by video-densitometry and corrected with β-actin values. As indicated by an asterisk, IGF I and IGFBP-5 mRNA levels from normal control rats were taken as 100%. Data are given as percentage of normal.

<table>
<thead>
<tr>
<th></th>
<th>normal</th>
<th>hypox</th>
<th>hypox + IGF I</th>
<th>hypox + GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF I</td>
<td>100*</td>
<td>37</td>
<td>64</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>9</td>
<td>47</td>
<td>279</td>
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<td></td>
<td>100*</td>
<td>12</td>
<td>32</td>
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<td></td>
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<td>9</td>
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<td>227</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>17 ± 13</td>
<td>42 ± 17</td>
<td>333 ± 107</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>100*</td>
<td>38</td>
<td>45</td>
<td>242</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>9</td>
<td>29</td>
<td>432</td>
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<td></td>
<td>100*</td>
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<td>51</td>
<td>536</td>
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<td>100*</td>
<td>13</td>
<td>43</td>
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<tr>
<td></td>
<td>100</td>
<td>17 ± 14</td>
<td>42 ± 9</td>
<td>375 ± 134</td>
</tr>
</tbody>
</table>
Figure 13: IGF I and IGFBP mRNA expression in epididymal adipose tissue. 20 μg of total RNA from normal (1), hypox (2), IGF I-treated (3) and GH-treated hypox rats (4) was separated on a 1% agarose gel, transferred to a nylon membrane and hybridized with rat IGF I, IGFBP-2 and human IGFBP-3 and -5 cDNAs.
3.5.3. White adipose tissue

The intensity of IGF I expression in WAT was comparable to that in liver (Figure 9). Like in liver, it was strictly regulated by GH (Figure 13 and Table 9). After hypophysectomy, IGF I mRNA levels decreased to 5% of normal, but they were nearly normalized by GH-infusion. In contrast to muscle, IGF I treatment had no significant effect on its own expression. The pattern of IGFBP-2 expression in WAT was unexpected: in contrast to liver, its signal was reduced after hypophysectomy and rose after GH-infusion. IGF I had no effect on WAT IGFBP-2 transcript levels of hypox rats. However, the low IGFBP-2 expression in WAT of hypox rats allowed no quantitative analysis by videodensitometry.

Table 9

IGF I, IGFBP-3 and -5 levels in WAT from 3 to 5 different experiments were analyzed by densitometry. Variations of gel loading were corrected by normalization with corresponding values obtained for β-actin. mRNA levels in normal WAT were taken as 100% (as indicated by the asterisk). Data are given as percentage of normal.

<table>
<thead>
<tr>
<th>adipose tissue</th>
<th>normal</th>
<th>hypox</th>
<th>hypox + IGF I</th>
<th>hypox + GH</th>
</tr>
</thead>
<tbody>
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<td>IGF I</td>
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<td>6.9</td>
<td>10.2</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>1.6</td>
<td>11.9</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>7.5</td>
<td>7.5</td>
<td>63</td>
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<td>100*</td>
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<td>6</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>3</td>
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<td>64</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.6 ± 2.5</td>
<td>8.5 ± 2.4</td>
<td>77 ± 24</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>100*</td>
<td>136</td>
<td>212</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>127</td>
<td>353</td>
<td>448</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>119</td>
<td>212</td>
<td>489</td>
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<td></td>
<td>100</td>
<td>127 ± 9</td>
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<td>IGFBP-5</td>
<td>100*</td>
<td>55</td>
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<td></td>
<td>100*</td>
<td>42</td>
<td>113</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>61</td>
<td>85</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>74</td>
<td>96</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>33</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>53 ± 16</td>
<td>88 ± 19</td>
<td>128 ± 22</td>
</tr>
</tbody>
</table>
The IGFBP-3 transcript levels in WAT were not influenced by hypophysectomy. IGF I-infusion led to a clear (2- to 3-fold) and GH treatment to an even more pronounced (5-fold) enhancement of IGFBP-3 expression. IGFBP-5 mRNA was strongly expressed in WAT (see also Figure 10). Signal intensity declined 50% after hypophysectomy. IGF I-infusion nearly restored and GH-treatment fully normalized IGFBP-5 mRNA levels.

3.5.4. Kidney

IGF I expression in kidney was low (see also Figure 9) and there was only a small reduction after hypophysectomy (Figure 14). IGFBP-2 mRNA was constitutively expressed at low levels. IGF I and IGFBP-2 transcript levels in kidney were too low for quantitative analysis. IGFBP-3 and -5 mRNAs were not IGF I- or GH-regulated, but their expression was higher than that of IGF I and IGFBP-2 (Table 10). Together with WAT, kidney was the organ with the strongest hybridization signal for IGFBP-5.

Table 10
IGFBP-3 and -5 mRNA levels in kidney. Northern blots were analyzed by videodensitometry from 3 or 4 experiments, respectively. Variations of gel loading were corrected by normalization with the corresponding β-actin values. Data are given in percentage of normal which was taken as 100% as indicated by an asterisk.

<table>
<thead>
<tr>
<th></th>
<th>normal</th>
<th>hypox</th>
<th>hypox + IGF I</th>
<th>hypox + GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-3</td>
<td>100*</td>
<td>112</td>
<td>115</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>108</td>
<td>98</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>114</td>
<td>87</td>
<td>81</td>
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<td></td>
<td>100</td>
<td>111 ± 3</td>
<td>98 ± 14</td>
<td>99 ± 15</td>
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<tr>
<td>IGFBP-5</td>
<td>100*</td>
<td>116</td>
<td>176</td>
<td>133</td>
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<tr>
<td></td>
<td>100*</td>
<td>129</td>
<td>186</td>
<td>154</td>
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<td></td>
<td>100*</td>
<td>87</td>
<td>88</td>
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</tr>
<tr>
<td></td>
<td>100*</td>
<td>116</td>
<td>122</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>112 ± 18</td>
<td>143 ± 46</td>
<td>117 ± 32</td>
</tr>
</tbody>
</table>
Figure 14: IGF I and IGFBP mRNAs in kidney, spleen and testes. 20 μg of total RNA from normal (1), hypox (2), IGF I-treated (3) and GH-treated hypox rats (4) was separated on a 1% agarose gel, transferred to a nylon membrane and hybridized with a cDNA probe specific for IGF I. The same filters were stripped and rehybridized with cDNA probes specific for IGFBPs.
3.5.5. Spleen

IGF I mRNA expression in spleen was little influenced by hypophysectomy (Figure 14). Together with WAT, it was the organ exhibiting the strongest hybridization signals for IGFBP-3, but in contrast to WAT, it was not influenced by hypophysectomy nor by GH-replacement.

3.5.6. Testes

IGF I expression in testes was lowered by 40% after hypophysectomy, nearly restored by IGF I- and fully restored by GH-treatment (Figure 14 and Table 11). Testes was a major organ of IGFBP-2 mRNA expression (Figure 10). It was expressed constitutively. The IGFBP-3 signal in testes from normal rats was weak and increased 9-fold in hypox controls. Neither IGF I- nor GH-infusion brought IGFBP-3 mRNA levels down to normal.

<table>
<thead>
<tr>
<th>Table 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF I, IGFBP-2 and -3 mRNA levels in testes of the four experimental groups were analyzed by densitometry. Mean values from 3 to 4 experiments are given in percentage of normal which were taken as 100% as indicated by an asterisk.</td>
</tr>
<tr>
<td>normal</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>IGF I</td>
</tr>
<tr>
<td></td>
</tr>
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<td></td>
</tr>
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<td>IGFBP-2</td>
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<tr>
<td></td>
</tr>
<tr>
<td>IGFBP-3</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
3.5.7. Brain

Analysis of total RNA from brain revealed low expression of IGF I (Figure 9). The signal in hypox animals was slightly higher than in normal rats. IGF I- and GH-infusion reduced IGF I transcript levels to normal (Figure 15 and Table 12). The regulation of IGFBP-2 mRNA in brain was inverse to serum IGFBP-2. It declined in hypox rats and rose in some of the rats after IGF I- and GH-treatment. IGFBP-5 mRNA expression in brain was increased 2-fold in hypox rats. IGF I- and GH-infusion both reversed enhanced expression to 131% and 122% of normal.

Table 12
IGF I, IGFBP-2 and -5 mRNA values in brain were determined by densitometric analysis of 4 to 5 Northern blot experiments with total brain RNA from different rats. Data are given as percentage of values from normal rats which were taken as 100% (indicated by an asterisk).

<table>
<thead>
<tr>
<th></th>
<th>normal</th>
<th>hypox</th>
<th>hypox + IGF I</th>
<th>hypox + GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF I</td>
<td>100*</td>
<td>110</td>
<td>84</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>262</td>
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<td>89</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>166 ± 84</td>
<td>89 ± 28</td>
<td>87 ± 6</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>100*</td>
<td>31</td>
<td>16</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>100*</td>
<td>35</td>
<td>36</td>
<td>38</td>
</tr>
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</tr>
<tr>
<td></td>
<td>100</td>
<td>40 ± 16</td>
<td>50 ± 30</td>
<td>57 ± 23</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>100*</td>
<td>244</td>
<td>120</td>
<td>122</td>
</tr>
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<td></td>
<td>100*</td>
<td>123</td>
<td>118</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>204 ± 56</td>
<td>131 ± 30</td>
<td>122 ± 35</td>
</tr>
</tbody>
</table>
Figure 15: IGF I and IGFBP mRNAs in brain and heart.
20 μg of total brain RNA from normal (1), hypox (2), IGF I-treated hypox (3) and GH-treated hypox rats (4) was subjected to Northern blot analysis. Hybridization was performed with IGF I, IGFBP-2 and -5 cDNA probes.
3.5.8. Heart

IGF I expression in heart muscle differed from that of skeletal muscle: Low IGF I mRNA levels were expressed in normal hearts (Figure 9). A 60% reduction of the hybridization signal was detectable in hypox rats with no change by IGF I-infusion (Figure 15 and Table 13). Heart muscle seemed to be less responsive to GH than skeletal muscle, since GH-infusion caused only a moderate increase of IGF I mRNA. Unlike in skeletal muscle, IGFBP-5 transcript levels in heart were slightly elevated above normal by hypophysectomy. IGF I-treatment induced a 2- to 2.5-fold stimulation of IGFBP-5 mRNA, which was not observed after GH-infusion. Only very low levels of IGFBP-3 mRNA were detectable in heart.

<table>
<thead>
<tr>
<th></th>
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<th>hypox + IGF I</th>
<th>hypox + GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF I</td>
<td>100*</td>
<td>40</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td></td>
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<td>64</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>32 ± 9</td>
<td>42 ± 13</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>100*</td>
<td>128</td>
<td>276</td>
<td>158</td>
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<td></td>
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<td>162</td>
<td>257</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>140 ± 28</td>
<td>246 ± 36</td>
<td>150 ± 17</td>
</tr>
</tbody>
</table>
Table 14

IGF I and IGFBP mRNA expression and their regulation in ten different rat tissues. Relative amounts of IGF I and IGFBP mRNAs are expressed by differently sized + symbols (estimated from Figures 9 and 10). GH-dependent regulation is indicated by shaded areas (light shadows: less than 50% decrease or increase after hypophysectomy as compared to normal; dark shadows: more than 50% decrease or increase).

1) stimulation/inhibition by IGF I and GH are in the same range; 2) GH-stimulation 1.5-fold higher than IGF I; 3) no decrease after hypophysectomy, but stimulation by IGF I and GH (2.5- and 5-fold); 4) stimulation by IGF I to 50 - 100% of normal, by GH to 350% of normal; 5) 40% increase after hypophysectomy, stimulation to 250% of normal by IGF I, no stimulation by GH above hypox level; 6) no stimulation by IGF I, normalization to 60-70% of normal by GH; 7) hybridization signal only detectable after hypophysectomy. 1-7 is based on results from Tables 6-13.

<table>
<thead>
<tr>
<th></th>
<th>IGF I</th>
<th>IGFBP-2</th>
<th>IGFBP-3</th>
<th>IGFBP-4</th>
<th>IGFBP-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>+</td>
<td>+ 6)</td>
<td>+ 7)</td>
<td>+ 1)</td>
<td>+ 1)</td>
</tr>
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<td>+ 4)</td>
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</tr>
<tr>
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<td></td>
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</tr>
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<td>+</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>WAT</td>
<td>+ 6)</td>
<td>+</td>
<td>+ 3)</td>
<td></td>
<td>+ 2)</td>
</tr>
</tbody>
</table>
3.4.9. Thymus

IGF I and IGFBP-3 mRNAs in thymus were very low and hardly visible after several days of exposure. No photography nor analysis by densitometry were possible.
4. DISCUSSION

The tissue distribution and regulation of the IGF I and IGFBP messages was studied in a hypox rat model. This model offered the possibility to compare systemic (IGF I-infusion) and systemic plus auto-/paracrine (GH-infusion) actions of IGF I, but its limitation was that direct IGF I-independent effects of GH could not be excluded. In addition, circulating IGF I in the two infusion conditions is not absolutely comparable: Endogenous IGF I induced by GH-infusion circulates predominately in the 150 kD IGFBP complex, whereas infused IGF I in hypox rats is mainly associated with the 50 kD IGFBP complex due to the lack of ALS (70). Hence, the capability of systemic IGF to cross the capillary barrier and reach its target tissues is different in IGF I- and GH-infused hypox rats.

With regard to growth parameters, IGF I-infusion mimicked all GH effects. IGF I stimulated skeletal growth less than GH, whereas its effects on kidney and spleen were greater than those of GH (see also [31]). At the level of IGF I and IGFBP gene expression, however, there were differences between IGF I- and GH-infusion depending on the type of tissue and IGFBP.

A striking difference between IGF I and GH was observed in liver regarding IGFBP-2 gene expression. The fact that IGF I-treatment resulted in 3-fold enhanced IGFBP-2 transcript levels but GH had no effect is unexplained. This IGF I effect must be indirect, because 1) hepatocytes belong to the few cell types lacking a functional type 1 IGF receptor (177) and 2) crossreaction of infused IGF I with the insulin receptor would suppress the IGFBP-2 message (118).

In contrast to IGF I and IGFBP-2, hepatic IGFBP-3 synthesis appears to be synthesized in non-parenchymal liver tissue, since cultured hepatocytes do not produce IGFBP-3 mRNA and protein (88). Hence, IGF I stimulates other cell types of the liver to produce "hepatic" IGFBP-3. Normalization of hepatic
IGFBP-3 mRNA levels by IGF I-infusion is in agreement with the reappearance of serum IGFBP-3 after IGF I-infusion into hypox rats (Figure 8 and [70, 178]). Both IGF I- and GH-infusion normalized hepatic IGFBP-3 and -4 gene expression, although local IGF I expression was completely suppressed by IGF I-, but restored by GH-infusion. These data are in favour of an endocrine mode of IGF action on IGFBP-3 and -4 expression in liver and speak against a direct GH-action.

A completely different situation is observed in skeletal muscle with respect to IGFBP-5. IGFBP-5 transcript levels correlated with local IGF I mRNA expression, which indicates that IGFBP-5 gene transcription is regulated by locally produced rather than systemic IGF I. The difference between soleus and gastrocnemius with respect to their sensitivity to hypophysectomy and IGF I-infusion is remarkable, but remains unexplained. However, both types of skeletal muscle exhibited a pronounced responsiveness to GH. The 3- to 4-fold overstimulation of IGF I and IGFBP-5 gene transcription by GH may be due to differences between the action of endogenous and exogenous GH. In this study, hypox rats received a slow, constant infusion of GH in contrast to the pulsatile secretion of endogenous GH in normal rats.

The observation that IGF I mRNA levels in WAT were in the same range as in liver was very surprising and prompted the studies reported in the following chapter. Like hepatocytes, adipocytes lack a functional type 1 IGF receptor (177). Therefore, it was not surprising that IGF I expression was normalized by GH-, but not by IGF I-infusion. But how can IGF I infusion stimulate IGFBP-3 transcript levels if the target cells lack the appropriate receptor? Liver and adipose tissue share the following features: as shown in Figures 9, 11 and 13, both tissues express high levels of IGF I mRNA which are tightly regulated by GH. Similar to liver, the gene expression and protein synthesis of IGFBP-3 in WAT may not occur in the adipocytes itself, but rather in stromal-vascular cells.
It is difficult to understand why IGFBP-3 mRNA expression in WAT remains unaffected by hypophysectomy but is enhanced by infusion of exogenous IGF I and GH. The previously mentioned differences between endogenous and exogenous IGF I and GH may cause this unexpected expression pattern. The IGFBP-5 transcript levels in WAT correlated more or less with serum IGF I. The better stimulation by GH-infusion probably reflects local plus systemic effects of GH on IGFBP-5 mRNA levels.

IGFBP mRNA expression in kidney, spleen and testes were unaffected by GH and IGF I. In contrast to Albiston and Herington who found increased renal IGFBP-3 expression in hypox rats (179), no change of renal IGFBP-3 transcript levels after hypophysectomy was observed in this study. In contrast to IGFBP-2, IGFBP-3 and -5 transcripts were markedly increased in testes of hypox rats with or without GH/IGF I-treatment. This may reflect the impaired maturation of testes in hypox rats or the lack of a pituitary-dependent suppressor of IGFBP-3 and -5 expression which is active in normal rats.

IGF I and IGFBP-2 and -5 expression in brain was completely inverse to what is observed in other tissues. The fact that this inverse regulation is valid for all components of the IGF/IGFBP-system expressed in brain may indicate a particular physiological relevance which is not yet understood.

In contrast to skeletal muscle, the IGFBP-5 message in heart increased only in response to IGF I- but not to GH-infusion. Apparently, GH exerted no direct nor indirect effects by local IGF I production. The response to IGF I-infusion may be due to the capability of IGF I circulating in the 50 kD complex to cross the capillary barrier.

Every tissue expressing IGF I mRNA also contained IGFBP mRNA. This suggests some connection in the regulation of their gene expression. The coproduction and secretion of IGF I and IGFBPs by the same cell may have an
important bearing on the regulation of IGF bioactivity near the sites of IGF production. This may be particularly important during rapid growth and differentiation such as embryogenesis or tissue regeneration, where tight regulation of IGF I action is required.

This study has provided information on the tissue specificity of endocrine and auto-/paracrine IGF I actions. GH effects on liver IGFBP-3 and -4 expression are mediated by endocrine rather than locally produced IGF I, whereas IGFBP-5 mRNA expression in muscle appears to be regulated predominantly by local IGF I. However, the interpretation of most of the data obtained with GH-infusion is difficult. By comparing IGF I- with GH-infusion, the relative contributions of endocrine IGF I versus locally produced IGF I and direct GH-actions cannot be clearly distinguished. For example, IGFBP-3 expression in WAT was stimulated to 500% of normal by GH. Circulating IGF I alone during IGF I-infusion (endocrine IGF I) caused a stimulation of 250% of normal. It is not clear, whether the additional 250% stimulation by GH was achieved by raising local IGF I expression or/and by direct GH-effects. Information about direct GH-actions could be achieved by co-infusion of GH with anti IGF I antiserum.

Another limitation of this study was that endocrine (circulating) IGF I in normal and in IGF I-infused hypox rats show an important difference: Hypox rats lack ALS, which is directly regulated by GH (70). Therefore, infused IGF I in hypox rats circulates as a 50 kD complex which can reach its target tissues (83) in contrast to the IGF I associated with the 150 kD complex in normal rats. ALS has been cloned recently (81) and mg quantities of recombinant ALS may become available soon. Co-infusion of IGF I and ALS into hypox rats would be interesting and could clarify whether the increased responsiveness of heart to
IGF I-infusion is dependent on the distribution of IGF I between the 150 kD and the 50 kD IGFBP complex.

In conclusion, infused IGF I in the absence of GH can exert different effects on IGF I and IGFBP expression than endogenous IGF I induced by GH-infusion. These differences can only be explained if one postulates an auto-/paracrine action of locally produced IGF I in addition to an endocrine action of circulating IGF I bound to serum IGFBPs. However, it is not clear whether the differences between the effects of IGF I- and GH-infusion on IGF I and IGFBP expression may also be due to direct (IGF I-independent) GH-action at the tissue level.
B. REGULATION OF IGF I AND IGFBPs BY GROWTH HORMONE IN RAT WHITE ADIPOSE TISSUE
1. INTRODUCTION

The understanding of differentiation processes is a major goal of cell biology. Various cell lines have been selected for their capability to differentiate in vitro. Differentiation of 3T3-L1 (180), 3T3-F442A (181) and OB1771 (182) preadipocytes had been classically achieved by the addition of isobutylmethylxanthine (MIX), dexamethasone and high doses of insulin or physiological concentrations of GH to serum containing media. In addition, Deslex et al. showed that primary rat preadipocytes undergo differentiation in defined serum-free medium supplemented with insulin, transferrin and triiodothyronine (T₃) (183). In 1988, Smith and coworkers reported that IGF I at physiological concentrations can substitute for insulin in 3T3-L1 cells (184). This was confirmed later in a serum-free 3T3-L1 cell system (185).

Although a role for IGF I in adipose conversion is generally accepted, few data on local IGF I expression and IGF I synthesis, IGFBP expression and their regulation in adipose tissue are available. No experiments investigating an auto-/paracrine role of IGF I in adipose tissue differentiation have been reported yet. However, studies using anti-sense oligonucleotides specific for IGF I mRNA have demonstrated that IGF I acts as an auto-/paracrine factor in muscle cell differentiation (35). A similar role of IGF I in adipose tissue is conceivable.
1.1. Aims of this study

In the course of the studies on tissue expression of IGF I and IGFBPs in rats, high levels of IGF I mRNA were demonstrated in white adipose tissue (WAT). This prompted me to investigate the regulation of IGF I gene expression by GH in vivo and in vitro. Furthermore, since IGF I action is modulated by IGFBPs, IGFBP mRNA expression in WAT and its regulation by GH in vivo was also analyzed.

Specifically, the following questions were addressed:

1) Does WAT contain only large amounts of IGF I mRNA or is this message effectively translated into protein?

2) Can IGF I mRNA and protein production be stimulated by GH in vitro?

3) Are IGFBPs expressed in WAT? Is their expression restricted to specific cell types within WAT?
2. MATERIAL AND METHODS

2.1. Animals

Groups of five hypox rats were infused with solvent, rhIGF I at a rate of 300 
µg/rat x day or rhGH at a rate of 200 mU (67 µg)/rat x day (see page 35). After 
six days of infusion, the rats were anesthetized and bled by aortic puncture. 
Epididymal fat pads were excised, blotted on a filter paper and weighed. They 
were immediately frozen in liquid nitrogen and stored at -80°C until RNA was 
isolated.

In short-term experiments, hypox rats were injected twice with 0.5 ml saline or 
100 mU rhGH at 900 h and 1700 h. 16 h later, the animals were sacrificed and 
epididymal fat pads were removed.

2.2. RNA-isolation and Northern Blotting

Total RNA was isolated according to standard procedures (175) (see page 37). 
Denatured RNA (20 µg) was electrophoresed on a 1 % agarose gel containing 
2 M formaldehyde, transferred onto a nylon membrane (Hybond-N, Amersham, 
UK), and RNA was fixed by UV-cross-linking. Hybridization was performed as 
described on page 41. The following cDNA probes were used: rat IGF I cDNA 
corresponding to the genomic sequences between nucleotide 2054 of exon 1 
and nucleotide 868 of exon 5 (57), rat IGFBP-2 cDNA (109) (rIGF I and 
rIGFBP-2 cDNAs were kindly provided by Dr. J. Schwander, Zentrum für Lehre 
und Forschung, Basel, Switzerland), human IGFBP-3 cDNA (cloned from a 
HepG-2 cDNA library, same nucleotide sequence as in [73]), human IGFBP-4 
cDNA (124), human IGFBP-5 cDNA (In the original publications IGFBP-4 had 
been named IGFBP-5 and IGFBP-5 had been named IGFBP-6. [134]) and rat
IGFBP-6 cDNA (kindly provided by Dr. S. Shimasaki, Whittier Institute for Diabetes and Endocrinology, La Jolla, CA) (135).

2.3. IGF I determination

Tissues were homogenized with a Polytron homogenizer in ice cold 0.1 M acetic acid containing 0.2 % human serum albumin (HSA), 1 μM pepstatin A, 0.3 trypsin inhibitor units/ml of aprotinin, 10 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride (all protease inhibitors were from Sigma [St. Louis MO, USA]). The homogenates were centrifuged at 16'000 x g for 10 min at 4°C. The fat layer was removed with a spatula and the infranatants were lyophilized. The lyophilized material was dissolved in 2 ml of 0.18 M ammonium acetate and centrifuged as above. Aliquots of the clear supernatants were lyophilized and used for IGF I determination.

Media obtained after in vitro incubations (see below) were dialyzed against 0.1 M ammonium acetate, pH 7.0 at 4°C before lyophilization. Lyophilized samples were dissolved in 300 μl H₂O, centrifuged at 16'000 x g for 10 min at 4°C and the supernatants were used for analysis of IGF I. Prior to radioimmunoassay (RIA), all samples were processed on Sep Pak C18 cartridges (Waters, Millipore, Milford MA, USA) according to the protocol supplied by Immunonuclear (Stillwater MN, USA) in order to remove IGFBPs. After reconstitution with 1 ml of phosphate-buffered saline/0.2 % HSA pH 7.4, samples were assayed at two or three different dilutions. Immunoreactive IGF I was determined by RIA (174) using a rabbit anti human IGF I antiserum at a final dilution of 1:10'000 and rat IGF I (gift from Dr. M. Kobayashi, Fujisawa, Japan) as a standard.
2.4. Analysis of IGFBPs by ligand and Western blotting

The method described in detail by Hossenlopp et al. (71) was used with slight modifications (70). Normal adult rat serum and concentrated tissue homogenates were electrophoresed on SDS/15% polyacrylamide slab gels under nonreducing conditions (except the $^{14}$C-labeled molecular weight marker: Rainbow Marker, Amersham, UK), transferred on nitrocellulose (ligand blot) or nylon membranes (Western blot, Hybond-C super, Amersham, UK) using a Transblot cell (Bio-Rad, Richmond CA, USA). Ligand blots were processed as described on page 36.

After overnight incubation with 1% fish gelatine in "saline" (page 36) at 4°C, the Western blots were incubated for 1 h with diluted rabbit antiserum specific for IGFBPs (anti rhIGFBP-2, kindly provided by Dr. D.R. Clemmons, Chapel Hill, NC, and anti rhIGFBP-4, both diluted 1:1000). Bound antibody was detected by a 2 h incubation with 1:1000 diluted second antibody (phosphatase conjugated goat anti-rabbit IgG, Sigma) and visualized using 5-bromo-4-chloro-3-indolylphosphate/ nitrobluetetrazoliumchloride (Sigma) as a precipitable substrate. Between all incubation steps, the filters were washed 2 x 15 min with "saline"/0.1% Tween 20 (Serva, Heidelberg, Germany) and 1 x 15 min with "saline".

2.5. Determination of blood volume in liver and WAT

The amount of blood in liver and adipose tissue extracts was determined according to (186). This method is based on the assumption that the proportion of oxyhemoglobin (HbO$_2$) in the circulating blood and the tissue capillaries is approximately the same.

Rat blood was collected into heparinized tubes and kept on ice. 0.1 ml of blood was diluted with 25 ml of H$_2$O, shaken and hemolyzed for 10 min on ice. Liver
and epididymal fat pads were excised and immediately homogenized in H₂O (about 5ml/g). The homogenates were centrifuged at 16'000 x g for 10 min at 4°C. The supernatants were transferred into fresh tubes and the pellets resuspended in 2 ml of H₂O and recentrifuged. The supernatants were pooled and kept on ice. The absorption spectra of blood and homogenate in several dilutions were recorded between 500 and 650 nm. The absorbance differences (ΔA_HbO₂ for tissue extracts and ΔA'_HbO₂ for blood samples) were determined by drawing a straight line between the two extinction maxima at 540 and 578 nm and a parallel line through the extinction minimum at 560 nm of the HbO₂ spectra obtained for the tissue extracts and the blood dilutions, respectively. The resulting absorbance difference is unaffected by turbidity and proportional to the HbO₂ content of the solution (186).

The fraction X of blood in the tissue was calculated as follows:

\[
X = \frac{\Delta A_{\text{HbO}_2} \times F_1 \times d_1}{\Delta A'_{\text{HbO}_2} \times F_2 \times d_2} \times 100 \text{ (%)}
\]

F₁ and F₂ are the dilution factors and d₁ and d₂ the respective light paths of the cuvettes.

2.6. Preparation of adipocytes and stromal-vascular cells

Epididymal fat pads from hypophysectomized and normal male Tif RAI rats were used. Adipocytes were obtained by collagenase digestion (type 1, from Clostridium histolyticum, Worthington, Freehold NJ, USA) of minced tissue (187). Tissue was digested for 45 min at 37°C in 6 ml of Krebs Ringer-HEPES buffer (131 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂ × 2 H₂O, 25 mM HEPES, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ x 7 H₂O) containing 5.6 mM D-glucose (AnalaR, BDH Chemicals Ltd, UK), 1% HSA and 6 mg collagenase/12 pads.
Cells were separated from tissue remnants by filtration through a piece of nylon stocking. The adipocytes were washed 3 times with Krebs Ringer-HEPES buffer by centrifugation at 150 x g for 30 s at 4°C. The cell pellet contained stromal vascular cells, endothelial cells and some erythrocytes. It was washed 3 times by centrifugation at 1'200 x g for 5 min at 4°C. Total RNA was isolated from both cell fractions.

2.7. In vitro incubations

Epididymal fat pads from hypophysectomized male Tif RAI rats were cut into small pieces. 4 pads per experiment were used for protein determination, 6 pads for RNA isolation. 4 h incubations were performed at 37°C in Krebs Ringer-HEPES buffer containing 5.6 mM D-glucose and 1 % HSA in a shaking water bath. Overnight incubations were done in F-12 media containing 0.1% bovine serum albumin and increasing rhGH concentrations at 37°C in an incubator (5% CO₂).
3. RESULTS

3.1. Expression and Regulation of IGF I in WAT in vivo

IGF I mRNA expression in WAT was determined by Northern blot analysis and compared with mRNA levels of various other organs (Figure 16). A major transcript with an estimated size of 7.5 kb was detected in all tissues. After long exposure, two minor bands at 1.6 - 1.7 kb and 0.8 - 1.1 kb appeared. In all organs and conditions the 7.5 kb band represented more than 90% of the total signal indicating that the isolated RNA was intact. IGF I mRNA levels in normal epididymal WAT were in the same range as in liver, the major source of circulating IGF I, and way above those in all other tissues analyzed.

![Figure 16: IGF I mRNA expression in various rat tissues. Total RNA isolated from different tissues of normal rats was subjected to Northern blotting using a probe specific for rat IGF I.](image-url)
Rat perirenal and human subcutaneous WAT contained significant levels of IGF I mRNA (Figure 17). In order to determine whether or not these high levels of gene transcripts are reflected by correspondingly high IGF I peptide levels, we measured IGF I in tissue homogenates. As shown in Table 15, epididymal fat pads from normal rats contain around 50 ng IGF I/g tissue. This is about 50% of the level in normal liver when expressed on a wet weight basis. However, if one considers that lipids make up 90% of the volume of adipose tissue, the IGF I concentration of WAT is about 5 times that of liver.

Figure 17: IGF I mRNA expression in human subcutaneous and in rat epididymal and perirenal WAT as compared to human and rat liver and human tumor tissue. Left panel: 10 μg of total RNA from human WAT (5) was compared to 20 μg of total RNA from human liver (1) and tumor samples (2-4: mesothelioma, mesenchymal fibrous tumor, leiomyosarcoma). Right panel: 20 μg of total RNA from rat epididymal (2) and perirenal WAT (3) were compared with rat liver (1) RNA.
IGF I mRNA and peptide content in WAT in vivo. IGF I mRNA levels in epididymal AT from normal, hypox, IGF I-infused and GH-infused rats were determined by Northern blotting. OD values of the 7.5 kb bands were measured by scanning densitometry and normalized against β-actin values. The IGF I mRNA levels of normal WAT were defined as 100%. Epididymal fat pads from normal, hypox, IGF I- and GH-treated hypox rats were homogenized and the IGF I content was measured by RIA (see Materials and Methods). IGF I mRNA levels are mean values obtained from five animals. IGF I peptide was measured in pools from two animals in each experiment and the data listed in the table are values from two separate experiments.* = measured against the human IGF I standard

Since IGF I mRNA in liver is regulated by GH (188, 189), we wondered whether the same holds true for WAT. To this end, hypophysectomized rats were infused for 6 days with or without rhIGF I or rhGH, and IGF I mRNA levels were determined in epididymal and perirenal fat. As shown in Figure 18, IGF I mRNA levels were decreased by 95% after hypophysectomy and restored to 80% of normal by GH infusion. In parallel, IGF I peptide content was decreased by 85% after hypophysectomy and again normalized by GH treatment (Table 15). Similar results were obtained with perirenal WAT (data not shown). These data suggest that hypophysectomy affected IGF I synthesis mainly at the transcriptional level. The induction of IGF I by GH treatment of hypox rats was observed already 24 h after 2 sc. injections of GH (2 x 100mU) (51.8 ng IGF I/g

<table>
<thead>
<tr>
<th>treatment</th>
<th>IGF I mRNA (%)</th>
<th>exp. 1</th>
<th>exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>100</td>
<td>46.3</td>
<td>53.9</td>
</tr>
<tr>
<td>hypox</td>
<td>5.0 ± 2.7</td>
<td>7.1</td>
<td>7.2</td>
</tr>
<tr>
<td>hypox + IGF I</td>
<td>9.4 ± 2.1</td>
<td>43.1*</td>
<td>62.6*</td>
</tr>
<tr>
<td>hypox + GH</td>
<td>80 ± 27</td>
<td>45.0</td>
<td>53.4</td>
</tr>
</tbody>
</table>
in WAT of GH-treated rats as compared to 19.5 ng IGF I/g in WAT of hypox controls). IGF I infusion had no significant effect on its own mRNA level. IGF I peptide levels in WAT after rhIGF I infusion were significantly higher than those in hypox control animals. However, the dilution curves in the RIA were parallel to the human rather than to the rat IGF I standard curves. This suggests that the immunoreactive IGF I measured in WAT during IGF I infusion is mostly due to human IGF I taken up from the circulation rather than to locally produced rat IGF I. This is also consistent with the unchanged mRNA level observed in this situation (Table 15).

Figure 18: Regulation of IGF I mRNA in epididymal WAT in vivo and in vitro. Hypox rats were infused for 6 days with solvent, rhIGF I (300 μg/day) and rhGH (200 mU/day). Total RNA was isolated from epididymal adipose tissue and compared with WAT from normal rats on a Northern blot (left panel). One representative experiment out of 5 is shown. Adipocytes and stromal-vascular cells were separated by collagenase digestion and total RNA was analyzed on a Northern blot (middle and right panel).
Since tissue IGF I correlated with IGF I serum levels in all situations studied, we wondered whether tissue IGF I may simply represent IGF I present in the blood of tissue capillaries. Therefore, we determined the blood fraction in liver and WAT: liver contained 2.03 ± 0.19 % and WAT 0.26 ± 0.08 % blood (mean values ± SD; n=6). Based on a mean hematocrit of 46%, the mean serum volume in 1 g of tissue was 10.80 ± 1.03 µl for liver and 1.42 ± 0.43 µl for WAT. Using a mean serum concentration of 1500 ng IGF I/ml in normal rats (Zapf et al., unpublished), the percentage of tissue IGF I that might stem from serum is 15.38 ± 1.49 % for liver and 4.33 ± 1.31 % for WAT.

3.2. Regulation of IGF I in WAT in vitro

Expression of IGF I mRNA in WAT of hypox rats increased in a dose-dependent manner after a 4 h incubation in the presence of increasing amounts of rhGH (Figures 19 and 20). The relative levels of IGF I mRNA were quantitated by scanning densitometry of the autoradiogram of the Northern blot, and all data were normalized with ß-actin mRNA. The value obtained for untreated control animals was taken as 1.0. Half-maximal stimulation of IGF I mRNA occurred between 0.25 and 0.5 nM GH (Figure 21). Maximal stimulation (4-5 fold) was achieved at 15 nM and after 2 h (Figure 19).

However, maximally stimulated IGF I mRNA levels in vitro were several-fold lower than those found in vivo (Figure 19). Although GH alone restored IGF I levels in vivo, we examined whether T3 was able to enhance the effect of GH in vitro. 2 nM T3 alone had no and in combination with 15 nM GH no further effect on IGF I mRNA (Figure 20).
Figure 19: Dose dependence of regulation of IGF I mRNA levels by GH in vitro. Epididymal fat pads of hypox rats were incubated for 2 and 4 h with GH at indicated concentrations. RNA was extracted and subsequently subjected to Northern blotting. Autoradiography of the Northern blot hybridized with an IGF I probe as well as with β-actin cDNA as a control.
Figure 20: Dose dependence of regulation of IGF I mRNA levels by GH in vitro. Epididymal fat pads of hypox rats were incubated for 4 h with GH at indicated concentrations. RNA was extracted and subsequently subjected to Northern blotting. Autoradiography of the Northern blot hybridized with an IGF I probe as well as with β-actin cDNA as a control. One experiment out of two with fat pads from 3 hypox rats per GH concentration is shown.
Figure 21: Dose dependence of regulation of IGF I mRNA levels by GH in vitro. The OD of the 7.5 kb bands from Northern blots of two experiments (one of which is shown in Figure 20) was determined by scanning densitometry. The data were corrected with β-actin and the value of the untreated hypox control was taken as 1. The bars show the upper and the lower value.

In contrast to IGF I mRNA, neither IGF I peptide levels in the tissue nor IGF I concentrations in the medium increased in response to GH in vitro (Table 16).
Table 16
IGF I concentrations (ng per g tissue) in tissue and media after incubation with various GH-concentrations in vitro. Incubations were performed in Krebs-Ringer-HEPES for 4 h (experiments 1 and 2) or in F-12 medium containing 1% bovine serum albumin for 18 h (experiment 3).

<table>
<thead>
<tr>
<th>condition</th>
<th>exp. 1</th>
<th>exp. 2</th>
<th>exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 nM GH</td>
<td>2.2</td>
<td>8.2</td>
<td>26.1</td>
</tr>
<tr>
<td>0.15 nM GH</td>
<td>15</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>1.5 nM GH</td>
<td>22</td>
<td>8.2</td>
<td>24.1</td>
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<tr>
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<tr>
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<td>2.1</td>
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</tr>
<tr>
<td>15 nM GH + 2 nM T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 nM T3</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 nM GH</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1500 nM GH</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 nM GH</td>
<td>7.1</td>
<td>8.4</td>
<td>9.2</td>
</tr>
<tr>
<td>0.15 nM GH</td>
<td>6.0</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>1.5 nM GH</td>
<td>7.3</td>
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</tr>
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<td>4.5 nM GH</td>
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<td>7.0</td>
<td></td>
</tr>
<tr>
<td>2 nM T3</td>
<td>8.3</td>
<td>8.1</td>
<td></td>
</tr>
</tbody>
</table>

3.3. Regulation of IGF I mRNA in adipocytes and stromal-vascular cells

To investigate whether IGF I gene expression in WAT is dependent on the cell type within WAT, stromal-vascular cells (adipose precursor cells) and adipocytes were prepared from WAT and RNA was isolated for Northern blot analysis. IGF I mRNA is present in both adipocytes and the stromal-vascular cells of normal rats (Figure 18, page 82). However, in contrast to the stromal-vascular fraction, IGF I mRNA had disappeared almost completely from adipocytes of hypox rats.
3.4. **IGFBP expression in WAT in vivo and in vitro**

To investigate which IGFBPs are expressed in WAT, the same Northern blot shown in Figure 18 was rehybridized with cDNAs specific for IGFBP-2 to -6 (Figure 22). Epididymal WAT contains five mRNA species of 1.35 kb, 1.7 kb, 2.2 kb, 2.5 kb and 6.5 kb coding for IGFBP-6, -2, -4, -3 and -5, respectively. IGFBP-2 mRNA levels decline after hypophysectomy and increase again after GH-treatment. IGFBP-3 expression is unchanged by hypophysectomy, but rises following IGF I- and even more after GH-treatment (Table 9, page 56). The IGFBP-4 mRNA level in WAT is very low and does not appear to be regulated by GH. IGFBP-5 and -6 messages are the two major IGFBP messages in WAT. They decrease after hypophysectomy and increase with GH but not with IGF I infusion.

Ligand blot analysis of WAT homogenate from normal rats showed the following IGF binding bands (Figure 23): A triplet at 42, 45 and 49 kD, a double band at about 33 and 32 kD, a band around 29 kD and one at 24 kD. Based on the comparison with rat serum, the triplet band of the WAT homogenate represents glycosylation variants of IGFBP-3 (70). Western blot analysis with specific antisera against rat IGFBP-2 and rhIGFBP-4 showed that the upper band of the 33/32 kD doublet corresponds to IGFBP-2 and the 29 kD band to IGFBP-4 (not shown). The 24 kD band probably corresponds to the non-glycosylated form of IGFBP-4 (139).
Figure 22: Expression of IGF I mRNA and IGFBP mRNAs in WAT. RNA isolated from WAT of normal (1), hypox (2), IGF I-infused hypox (3) and GH-infused hypox (4) rats was hybridized with cDNA probes specific for IGF I (panel A) and for IGFBP-2, -3, -4, -5 and -6 (panel B). One representative result of 3-5 different rats is shown.
Figure 23: $^{125}$I-IGF II ligand blot analysis of serum, liver and WAT homogenates. Two microlitres of normal adult rat serum (S), 20 microlitres of WAT homogenate (AT) (52 mg tissue) and 20 microlitres of liver homogenate (L) (11 mg tissue) were analyzed on a ligand blot.
IGFBP-2, -3 and -5 mRNAs in hypox WAT were not affected by GH in vitro (Figure 24).

**Figure 24**: Expression of IGFBP-2, -3 and -5 mRNAs in WAT in vitro. The same filter shown in Figure 20 was stripped and rehybridized with cDNA probes specific for rat IGFBP-2 and human IGFBP-3 and -5.
3.5. Cell-specific expression of IGFBP mRNAs

In contrast to IGF I, which is expressed in both adipocytes and adipose precursor cells, IGFBPs are differentially expressed (Figure 25). IGFBP-2 and -3 mRNAs are restricted to stromal-vascular cells whereas IGFBP-5 mRNA is detectable in both cell types. IGFBP-2 and -3 mRNA are not detectable in adipocytes of normal and hypox rats. They are expressed in stromal-vascular cells of normal rats, decrease after hypophysectomy (19 ± 11% and 49 ± 12% of normal, respectively, n=2) and increase again after GH-treatment (74 ± 24% and 189 ± 31% of normal, respectively, n=2). IGFBP-5 mRNA is found predominantly in stromal-vascular cells and less in adipocytes (30 ± 2% of the level in stromal-vascular cells, n=2). GH-treatment raises IGFBP-5 mRNA only in stromal-vascular cells (from 36 ± 6% to 89 ± 4%, n=2).
Figure 25: Cell specific expression and regulation of IGFBPs in WAT. Epididymal adipose tissue was digested with collagenase and adipocytes and stromal-vascular cells were separated by centrifugation as described in Materials and Methods. Total RNA of adipocytes (A) and stromal-vascular cells (S) from normal, hypox and GH-treated hypox rats (2 s.c. GH injections) was hybridized with cDNA probes specific for IGF I, IGFBP-2, -3 and -5. One of two experiments is shown.
4. DISCUSSION

4.1. The messages of IGF I and IGFBPs in white adipose tissue (WAT)

It may not be surprising that WAT like many other tissues (22, 170) produces IGF I and IGFBPs. However, the high level of IGF I mRNA and IGF I peptide and the special expression pattern of IGFBPs in this tissue are striking. Thus, the IGF I mRNA levels are similar to those found in liver, the major source of serum IGF I (21) and they lie far above the levels of other tissues. Similarly, significantly greater amounts of IGF I peptide are extractable from WAT than from other tissues on a tissue wet weight basis (22). Only liver contains more, i.e. twice as much as WAT. Since more than 90% of the adipose tissue wet weight consists of lipids, the estimated IGF I concentration in the cytosol and interstitial fluid of WAT is around 500 ng/ml, i.e. several-fold higher than in liver. In contrast to the liver (page 51), WAT expresses significant levels of IGFBP-5 mRNA which is present in both adipocytes and stromal-vascular cells.

The high expression level of IGF I mRNA and the high IGF I concentration in WAT raise questions about the physiological roles of IGF I in this tissue. Several possibilities can be envisaged: 1. IGF I from WAT might contribute to the IGF I levels in the circulation. 2. It might exert acute insulin-like effects and thus contribute to GH-regulated glucose metabolism in the adipocyte. 3. It might enhance proliferation and differentiation of adipose precursor cells into adipocytes.

Studies in the isolated perfused rat liver have shown that the secretion rate of IGF I into the perfusate fully accounts for normal serum IGF I levels in the rat (21). Unlike the perfused rat liver, WAT in vitro does not release substantial amounts of IGF I into the medium (see Table 16). Therefore, it appears unlikely that WAT contributes significantly to serum IGF I. Rather IGF I produced in WAT acts locally in an auto/paracrine manner.
Acute insulin-like effects of IGF I on adipocytes are mediated via the insulin receptor (190, 191). Furthermore, type 1 IGF receptors are not detectable in rat adipocytes (177). Stimulation of glucose oxidation and lipid synthesis from glucose in WAT occurs over a concentration range of 10 to 100 ng/ml (1.33 - 13.3 x 10^{-9} M) of IGF I (192). Therefore, a concentration of 500 ng IGF I/ml water in the cytosolic plus interstitial space of adipose tissue would cause a constant maximal stimulation of glucose metabolism and would no longer allow insulin, the main regulator of glucose entry into the fat cell, to exert any additional modulatory effect. It is, therefore, essential to include IGFBPs in these considerations. IGFBPs inhibit the acute insulin-like effects of IGF I on the adipocyte (193). Although the concentration of free IGF in WAT is unknown, we can assume that IGFBP-2 to -6 bind a major portion of the IGF I in this tissue and thus prevent its insulin-like actions.

In contrast to the adipocyte, adipocyte precursor cells do contain type 1 IGF receptors (185). The affinity (K_D value) of IGF I for the type 1 IGF receptor is 1.5 x 10^{-9} M (44). IGF I effects mediated via the type 1 IGF receptor are usually observed over a concentration range of 10^{-10} to 10^{-8} M. Despite binding of IGF I to IGFBPs, the free IGF I concentration in the interstitial fluid of WAT may still be high enough to allow interaction with the type 1 IGF receptor on adipose precursor cells. In addition, the bioavailability of IGF I to the type 1 IGF receptor may be influenced by BP-specific proteases which cause release of IGF I from the BP-IGF I complexes upon degradation of the BP (194, 157). Thus, the interaction of IGF I with the type 1 IGF receptor in adipose precursor cells might trigger differentiation.
4.2. Regulation of IGF I and IGFBPs by GH

According to the somatomedin hypothesis (3), GH stimulates IGF I production by the liver. This leads to an increase of IGF I in the circulation. In addition, GH also regulates local IGF I production in other tissues. Green et al. have postulated that during differentiation of 3T3 cells into adipocytes GH acts at early stages of differentiation and that its subsequent effects on "clonal expansion" are mediated by IGF I (195). In this context, we studied the effect of GH on both IGF I and IGFBP mRNA levels in vivo and in vitro. Like in liver, IGF I mRNA and IGF I peptide levels were drastically decreased in epididymal and perirenal adipose tissue of hypophysectomized rats. GH replacement restored the IGF I mRNA level nearly completely and led to normalization of the IGF I peptide level in this tissue. Thus, GH appears to be the main regulator of adipose tissue IGF I.

The in vivo regulation of IGF I mRNA in WAT by GH has recently been studied by Vikman et al. (196). These authors also found decreased IGF I mRNA after hypophysectomy and an increase during GH replacement, although the decrease of the IGF I message after hypophysectomy was not as pronounced as in our animals.

GH-dependence of IGFBP-3 serum levels is well established (70, 197). Our studies show that the expression of IGFBP-2, -3, -5 and -6 in WAT is enhanced by GH, but not by IGF I. The localization of IGFBP-3 in stromal-vascular tissue is consistent with the finding that IGFBP-3 is produced by vascular endothelial cells (89). In these cells, like in serum, IGFBP-3 is regulated by GH. In WAT, GH appears to stimulate IGFBP-2 to -6 mRNA directly and not via IGF I.

The high level of IGF I and its regulation by GH in WAT together with the well established notion that IGF I is a differentiation factor (34, 35, 198) suggest that IGF I is also important for fat cell differentiation and adipose tissue growth. Differentiation of adipose precursor cells is classically achieved in a medium
containing pharmacological concentrations of insulin besides dexamethasone and isobutylmethylxanthine. Replacement of the high insulin doses by low doses of IGF I (10 nM) results in the same adipose conversion efficiency (184). Ailhaud et al. proposed a model for adipose conversion in which a combination of signals, each present at threshold levels, is required to trigger the critical mitosis leading to terminal differentiation (36). This combination consists of the cyclic AMP-, the diacyl glycerol-, and a third pathway triggered by insulin or IGF I. We, therefore, suggest, that IGF I produced locally under the influence of GH plays a critical role in adipocyte differentiation, which is modulated by the IGFBPs of WAT.
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