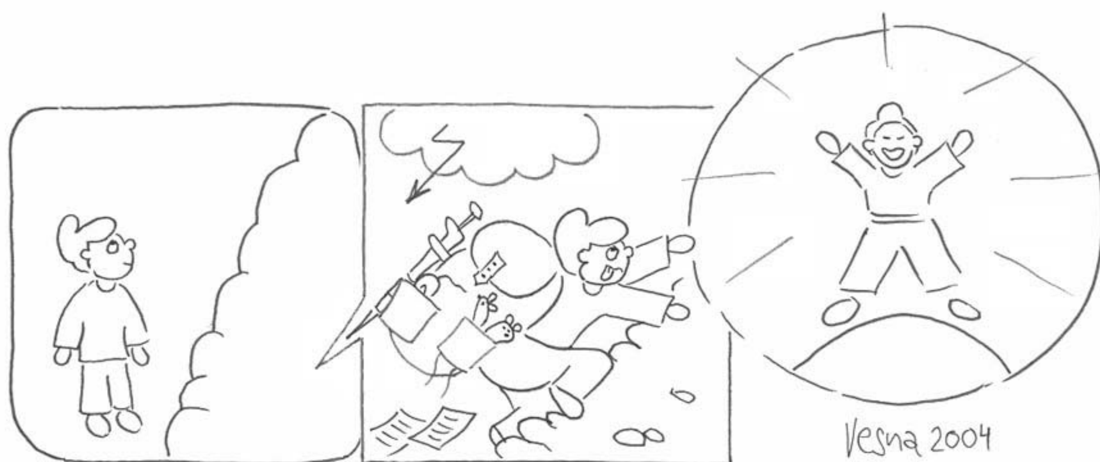
The GH/IGF axis in the mouse kidney

Vesna Cingel-Ristić

The journey is more important than the destination.
…although it helps if you know where you want to go…



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The GH/IGF axis in the mouse kidney

De GH/IGF as in de nier van de muis

PROEFSCHRIFT

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We dance round in a ring and suppose,
But the Secret sits in the middle and knows.

Robert Frost

Mami i tati

Mojim sunašcima Dejanu i Milošu

Contents

List of abbreviations……………………………………………………………..….8

Chapter 1 **General Introduction**……………………………………………...…11
The GH/IGF axis……………………………………………………….…13
Outline of this thesis ……………………………………………………..…18

Chapter 2 **The physiological and pathophysiological roles of the GH/IGF-axis in the kidney: Lessons from experimental rodent models**...21

Chapter 3 **Kidney growth in normal and diabetic mice is not affected by human insulin-like growth factor binding protein-1 administration**……………………………………………………………37

Chapter 4 **Dose-response effects of a new growth hormone receptor antagonist (B2036-PEG) on circulating, hepatic and renal expression of the growth hormone/insulin-like growth factor system in adult mice**…………………………………………….....57

Chapter 5 **High-protein induced renal enlargement is growth hormone independent**……………………………………………………………..69

Chapter 6 **Administration of human insulin-like growth factor binding protein-1 increases circulating levels of growth hormone in mice**……………………………………………………………………..81

Chapter 7 **General Discussion**…………………………………..………..……..91

Chapter 8 **Summary**……………………….……………………………………103

Summary in Dutch (Nederlandse samenvatting)……………….….…106

Summary in Serbian (Pregled na srpskom)……………………….....108

Curriculum vitae…………………………..………………………………….…....110

List of publications……………..……………………………………..…………....111

And in the end………………………………………………………………….…112

List of abbreviations

ALS	acid labile subunit
ANOVA	analysis of variance
BGL	blood glucose levels
BSA	bovine serum albumin
BW	body weight
cDNA	complementary deoxyribonucleic acid
CRF	chronic renal failure
CRG	compensatory renal growth
CV	coefficient of variance
dNTP	deoxyribonucleoside triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked iImmunosorbent assay
FC	food consumption
FPLC	fast performance liquid chromatography
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFR	glomerular filtration rate
GH	growth hormone
GHBP	growth hormone binding protein
GHR	growth hormone receptor
GHRA	GHR antagonist
GHRH	growth hormone releasing hormone
HP	high-protein
HPRT	hypoxanthine phosphoribosyltransferase
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGF-IR	IGF-I receptor, type 1 IGF receptor
IGF-II/MP6R	IGF-II/mannose-6-phosphate, type 2 IGF receptor
Jak	Janus kinase
kDa	kilo Dalton
KO	knock-out
KW	kidney weight
LP	low-protein
LSD	least significant difference
LW	liver weight
MAP	mitogen activated protein
mRNA	messenger ribonucleic acid
mTAL	medullary thick ascending limb
NO	nitric oxide
NOD	nonobese diabetic
NP	normal-protein, also **SP**
PAGE	polyacrilamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor

PEG	polyethylene glycol
PI 3	phosphatidyl-inositol-3
RIA	radioimmunoassay
RGD	arginine-glycine-aspartic acid
RPF	renal plasma flow
RT	reverse transcription
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SP	standard-protein, also **NP**
SS	somatostatin
Stat	signal transducers and activators of transcription
STZ	streptozotocin
TBS	Tris buffered saline
TG	transgenic
TGF	transforming growth factor
UAE	urinary albumin excretion
VEGF	vascular endothelial growth factor
WIB	Western immuno blot
WLB	Western ligand blot

Chapter 1

General Introduction

THE GH/IGF AXIS

Growth hormone (GH) is a protein hormone synthesized and secreted by somatotroph cells within the anterior pituitary predominantly under regulation of hypothalamic peptides, GH releasing hormone (GHRH) and somatostatin (SS) (1-3) (**Figure 1**). Further, production of GH is modulated by various neuronal and endocrine factors. Genetic predisposition, nutrition, stress, exercise and sleep pattern are all known to influence GH release. GH itself can regulate its own production at the hypothalamic level, where it modulates the release of GHRH and SS (2), and at pituitary level where it has autocrine inhibitory effect on secretion from the somatotroph (4) (**Figure 1**). GH secretion is also regulated through a negative feedback loop involving the principal mediator of GH activity, insulin-like growth factor (IGF-I) (5) (**Figure 1**). High serum levels of IGF-I are believed to decrease GH secretion not only by directly suppressing the somatotroph, but also by stimulating release of somatostatin from the hypothalamus. However, recent results from liver-specific IGF-I KO mouse suggest that IGF-I feedback regulates GH secretion at the pituitary rather than at the hypothalamic level (6). Integration of all the factors that affect GH synthesis and secretion results in a pulsatile pattern of release.

The multiple actions of GH are initiated by binding to the GH receptor (GHR), a single-pass transmembrane glycoprotein located in the plasma membrane of the target cells (7). The GHR belongs to a large superfamily of cytokine/hematopoietin receptors which share some structural motifs and whose signal transducing processes utilize Jak-Stat pathway (8). A single GH molecule binds sequentially to two receptor molecules, forming an active complex (9) (**Figure 1**). Intracellular events following activation of GHR include activation of associated tyrosine kinase, Janus kinase (Jak-2), which leads to phosphorylation and activation of numerous cytoplasmic proteins including the signal transducers and activators of transcription (Stat) proteins, as well as adaptor proteins leading to the activation of the mitogen activated protein (MAP) kinase and the phosphatidyl-inositol-3-kinase (PI 3-kinase) pathways (10).

In serum, GH can be bound to high-affinity GH-binding protein (GHBP), a circulating glycoprotein that binds the hormone with affinity comparable to that of the receptor (11). It may dampen pituitary-secretion derived GH oscillations and prolongs its half-life in the circulation. In all species, GHBP corresponds to the extracellular hormone-binding domain of GHR and is a product of the same gene, although its generation differs between species. In human and many other species generation of GHBP involves proteolytic cleavage of the full length GHR protein (12), whereas in mice and rats distinct GHR- and GHBP- encoding mRNAs are derived from a single gene by alternative splicing (13-15).

GH has major role in control of several complex physiologic processes, including growth and metabolism (3, 16). It exerts its physiologic effects either directly through GHR on target cells or indirectly through the IGFs (IGF-I and IGF-II) and in some cases it appears that both direct and indirect effects are at play (3, 17). In addition to its major involvement in growth promotion, GH has important effects on protein, lipid and carbohydrate metabolism (16, 18).

IGF-I is a single chain polypeptide growth factor that shares sequence identity with pro-insulin (50%) and IGF-II (70%) (19, 20). IGF-I is synthesized and released from multiple tissues, although the hepatic biosynthesis accounts for the major portion of

circulating IGF-I (21). In addition to the initially observed endocrine action, IGF-I is acting in an autocrine/paracrine manner (3, 22, 23). However, the strong GH-dependence of hepatic IGF-I expression is unique. Renal IGF-I expression is only partially GH-dependant while expression in most other non-hepatic tissues is GH-independent (17, 24).

Both IGF-I and IGF-II play essential roles in growth and development as demonstrated by null mutation experiments (25-28). Mice with inactivated IGF-I are growth retarded and mostly die after birth. Those that survive are severely growth retarded, have developmental defects in multiple organs and are infertile. IGF-II null mice, although small at birth, grow normally postnatally. Thus, while IGF-I is important both in prenatal and postnatal growth and development, IGF-II is primarily responsible for embryonic growth (26). Following birth, serum IGF-II levels decrease significantly in the mouse. Mice lacking both IGF-I and IGF-II action exhibit even lower birth weight than single IGF KO (28). Surprisingly, liver IGF-I deficient mice grow normally although their serum IGF-I levels are down to 20% of normal (22, 23). It appears that liver-derived IGF-I is not required for postnatal growth, which presents major modification to the classic somatomedin hypothesis (3).

IGF-I binds with high affinity to IGF-I receptor (IGF-IR, also known as type 1 IGF receptor), with lesser affinity to IGF-II/mannose-6-phosphate (IGF-II/M6PR, also known as type 2 IGF receptor) and with much lesser affinity to insulin receptor. IGF-IR is a heterotetrameric transmembrane glycoprotein, which mediates most of the biological effects of IGFs. The IGF-IR, like the insulin receptor, has intrinsic tyrosine kinase activity which upon ligand binding activates second messenger systems within the target cell through a protein phosphorylation cascade (29, 30). Mice with complete disruption of IGF-IR die soon after birth due to respiratory failure (27, 28) and it was only with conditional gene targeting, it became apparent that ubiquitous downregulation of IGF-IR has effects exclusively on postnatal somatic growth (31). Since IGF-II/IGF-IR double KO exhibited greater growth retardation than IGF-IR KO, it became apparent that another receptor apart from IGF-IR mediates growth promoting actions of IGF-II, later identified to be insulin receptor (28, 32, 33).

IGF-II/M6PR is monomeric receptor without tyrosine kinase activity functioning also as the cation–independent mannose-6-phosphate receptor (34). Complete KO of IGF-II/M6PR causes fetal overgrowth accompanied by severe organ abnormalities and neonatal lethality, while tissue-specific inactivation gives rise to viable animals opening possibility to study role of this receptor in different tissues (35, 36). Classical IGF-II/M6PR-deficient mice can be rescued by concomitant deficiency in IGF-II or IGF-IR pointing out that failure to degrade IGF-II and the subsequent excess signaling through IGF-IR gives rise to the lethal phenotype (35). Thus, IGF-II/M6PR is involved in internalization and degradation of IGF-II, thereby reducing IGF-II levels during fetal development.

In the circulation, only a minor fraction of IGFs are present in the free form (37). More than 99% of serum IGF-I is bound to 6 high-affinity IGF binding proteins (IGFBPs). IGFBPs transport IGFs in the circulation and prolong their half-life by protecting them from proteolytic degradation, buffer their potential hypoglycemic effects and regulate their extravascular distribution (38, 39). IGFBP-3 is the most abundant IGFBP in the circulation. The acid labile subunit (ALS) combines with IGFBP-3 or -5 to form abundant and stable heterotrimeric IGF-I transporting

complexes which are confined to the circulation (40). Most target tissues also express IGFBPs where they further regulate the local actions of IGFs. IGFBPs contain different motifs that mediate their binding to cell surface and extracellular matrix (ECM) proteins (41) (**Figure 2A**). IGFBP-1 and IGFBP-2 both contain an Arg-Gly-Asp tripeptide (RGD sequence) near the C-terminal, which enables them to interact with integrins, a large family of cell adhesion receptors involved in cell-cell and cell-ECM interactions. IGFBP-3, -5 and –6 posses highly basic heparin-binding motif which can associate with glycosaminoglycan-containing molecules, such as proteoglycans on cell surfaces and ECM. Through these interactions IGFBPs could facilitate the delivery of IGF-I to adjacent IGF-I receptors, enhancing the subsequent binding and actions of IGF-I on cells (**Figure 2B**). However, IGFBPS may under certain conditions inhibit IGF action by forming inactive complexes with IGFs. Such opposing roles have been described for IGFBP-1, -3 and -5 (42). Differential post-translational modifications of IGFBPs can alter their biological activity. In case of human IGFBP-1, differential phosphorylation accounts for its dual effect on IGF-I mediated events (43), whereas phosphorylation does not affect rat IGFBP-1 affinity for IGF-I (44). IGFBP/IGF regulation is further complicated by specific proteases that proteolyse IGFBPs providing a mechanism for the acute regulation of IGFBP degradation and alteration of their IGF binding affinity (45, 46). It has been increasingly recognized that IGFBPs not only regulate IGF bioavailability but also may have intrinsic biological activity independent of IGF, affecting cell motility and adhesion, cell cycle and apoptosis (41, 46) (**Figure 2B**). Since IGFBPs share high inter- and intra- species protein sequence homology (38), functional compensation within this family of related proteins suggested by KO experiments is not surprising. Genetic ablation of IGFBP-2, apart from restricted alterations in organ weights, did not affect neither prenatal nor postnatal growth (47). While GH and IGF-I in IGFBP-2 mutant serum were not significantly changed, elevated IGFBP-1, -3 and –4 suggested functional redundancy in the IGFBP family.

In summary, multifunctional IGFBPs play diverse and important roles in the extracellular regulation of IGF activity as they differ in their tissue distribution, binding affinity for the IGF-I, regulation and degradation. Furthermore, as most cells express more than one IGFBP, regulation of each one of them plays an important role in regulating cellular processes, via modulating IGF-IR activation by IGFs or via IGF-independent mechanism. As will be discussed below, the GH/IGF axis, including IGFRs and IGFBPs, is altered in mouse models of kidney growth. The significance of these changes is still largely unknown. Further refinement in temporal and spatial IGFBP deletion or expression, involving mouse models with alterations in multiple IGFBPs and responsiveness of these models to physiological influences are expected to provide precise conclusions on IGFBP action in future.

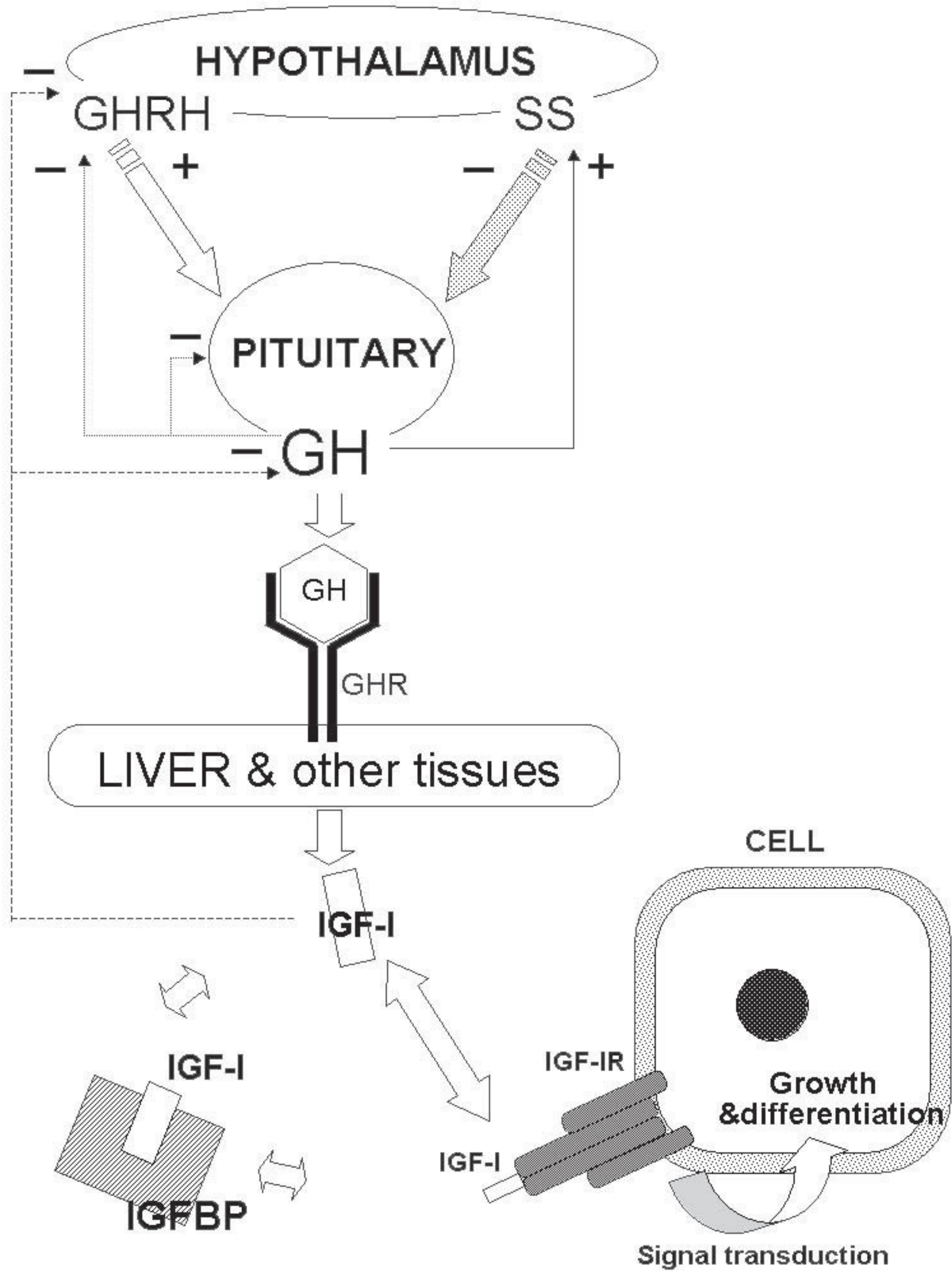

Figure 1.
Schematic representation of the growth hormone (**GH**)/ insulin-like growth factor (**IGF**) axis. **GHRH**, GH releasing hormone, **SS**, somatostatin, **GHR**, GH receptor, **IGF-IR**, IGF-I receptor, **IGFBP**, IGF binding protein, + indicates stimulation, - indicates inhibition

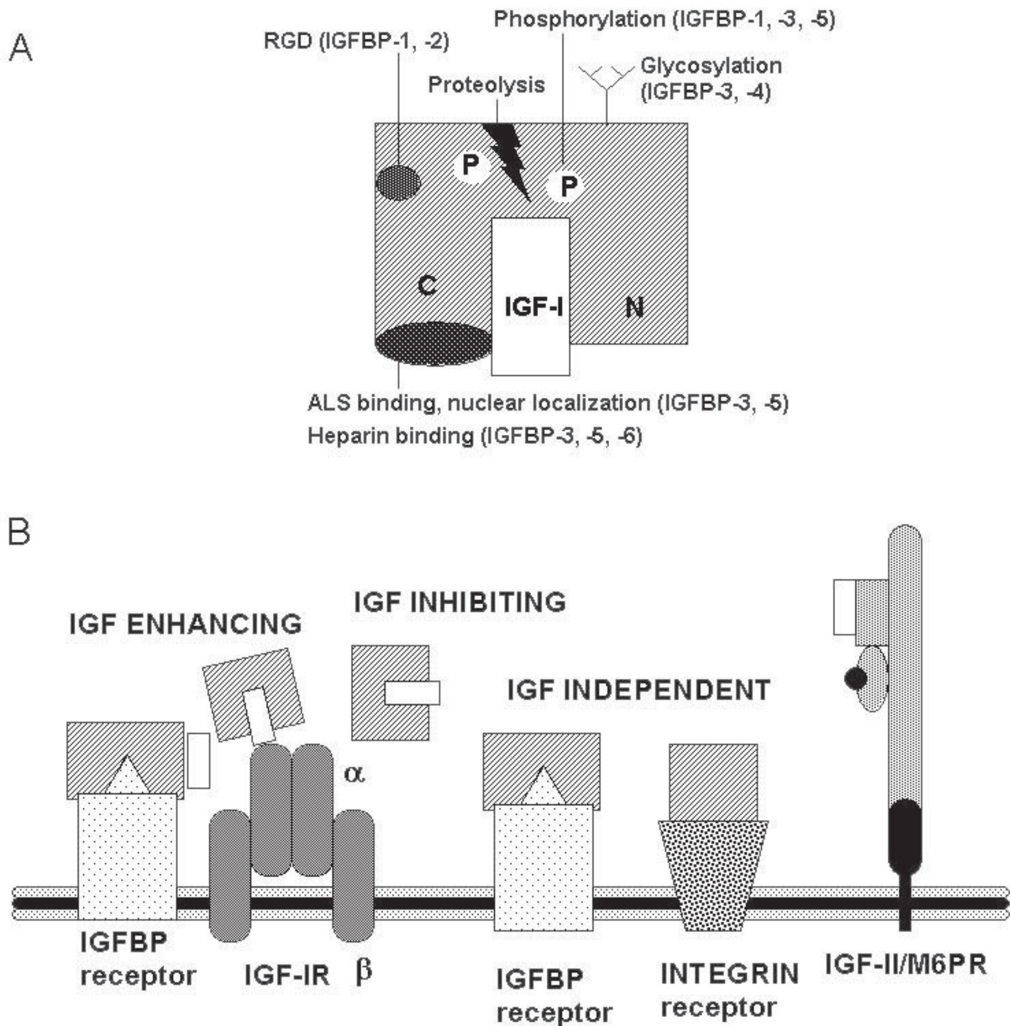

Figure 2.
Schematic illustration of insulin-like growth factor binding protein (IGFBP) structure showing functional domains and sites of posttranslational modification (A) involved in IGF-dependent and IGF-independent actions (B), modified from ref. 39, 41

OUTLINE OF THIS THESIS

The GH/IGF axis has been postulated as pathogenic factor in several forms of renal growth, including those induced by diabetes and high-protein (HP) diet. Using mouse models, we aimed to further elucidate the role of GH/IGF axis in the renal changes following onset of diabetes and initiation of HP diet.

Next chapter gives an overview of the GH/IGF system expression in the mouse kidney, as well as their involvement in renal changes taking place after induction of diabetes, nephrectomy and initiation of HP diet. Chapters 3-6 describe various experimental studies.

Chapter 3 describes efforts to elucidate mechanisms leading to early renal changes in experimental diabetes, i.e. increased kidney weight, increased glomerular volume and albuminuria. The contribution of the circulatory and the local renal IGF system is investigated and the hypothesis that IGFBP-1 captures IGF-I in the kidney is tested.

The effects of GH in regulating circulating, hepatic and renal expression of the GH/IGF system in the mouse are studied by administering a novel GH receptor antagonist (GHRA) at different doses, establishing a dose-response curve, given in **Chapter 4.**

In **Chapter 5** we examine the effect of this GHRA on renal growth and renal GH/IGF-system expression in HP-fed mice to elucidate a possible direct role for GH in HP-induced renal growth.

GH secretion is regulated through a negative feedback loop involving the principal mediator of GH activity, IGF-I. Several studies indicate that IGFBP-1 is able to alter GH secretion via IGF-I feedback mechanism. Potential direct influence of IGFBP-1 on GH secretion and the effect of hIGFBP-1 administration on gene expression of IGFBP-1 and IGF-I and their protein levels in the mouse liver and kidney are explored in **Chapter 6**.

Results from all the studies are summarized and discussed in **Chapters 7** and **8**.

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Chapter 2

The physiological and pathophysiological roles of the GH/IGF-axis in the kidney: Lessons from experimental rodent models

Cingel-Ristić V, Flyvbjerg A , Drop SLS

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Review

The physiological and pathophysiological roles of the GH/IGF-axis in the kidney: Lessons from experimental rodent models

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Abstract

The growth hormone (GH)/insulin-like growth factor (IGF) system plays an important role in renal development, growth, function and pathophysiology. IGF-I has been associated with renal/glomerular hypertrophy and compensatory renal growth. Potential effects on glomerular size are of interest, since an increase in glomerular size may be permissive for the development of glomerulosclerosis. In an effort to abolish the decline of renal function and possibly to restore the renal structure, different approaches have been tested in experimental models of nephropathy, focusing mainly on early renal changes. The involvement of the GH/IGF system in renal pathophysiology has been studied in much detail in the rat. In view of the growing interest in murine physiology, occurring in large part by genetically modified animals, this review examines those aspects of GH, IGFs, their receptors and binding proteins that relate both to mouse kidney physiology and to a number of conditions characterized by pathophysiological renal changes. A deeper understanding of the role of the GH/IGF system in renal dysfunction may stimulate the development of novel therapeutic approaches aiming at preventing or retarding various kidney diseases.
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Keywords: Growth hormone; Insulin-like growth factor; Kidney; Diabetes; Nephrectomy; High-protein diet

1. Introduction

Progression of renal disease, irrespective of the primary cause, is characterized by deterioration of renal structure and function ultimately leading to end-stage renal disease (ESRD). The available treatments for chronic renal failure, being either dialysis or kidney transplantation, make ESRD one of the most expensive diseases to treat in the developed world [1]. The involvement of the growth hormone (GH)/ insulin-like growth factor (IGF) axis in renal complications, particularly in diabetes, has been recognized for a long time [2,3].

GH exerts its physiologic effects either directly and/or indirectly through the insulin-like growth factors (IGFs, i.e., IGF-I and IGF-II) [4,5]. The hepatic biosynthesis accounts for the major portion of circulating IGF-I, but IGF-I is synthesized and released from multiple tissues where it can act in an autocrine/paracrine manner [4,6]. However, the strong GH-dependence of hepatic IGF-I expression is unique. Renal IGF-I expression is only partially GH-dependent while expression in most other non-hepatic tissues is GH-independent [5,7].

The major fraction of serum IGF-I is bound to six high-affinity IGF binding proteins (IGFBPs), which transport IGFs in the circulation and prolong their half-life by protecting them from proteolytic degradation, buffer their potential hypoglycaemic effects and regulate their extravascular distribution [8–10]. Most target tissues also express IGFBPs where they further regulate the local actions of IGFs or act independent of IGF, affecting cell motility and adhesion, cell cycle and apoptosis [11,12]. Since IGFBPs share high inter-

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and intra-species protein sequence homology [10], functional compensation within this family of related proteins suggested by knockout (KO) experiments is not surprising [13].

Involvement of the GH/IGF-system in the normal and the diseased kidney has been the topic of previous reviews. However, these reviews have predominantly been based on data obtained in rats and humans [14,15]. The aim of the present review is to focus on the emerging amount of data in support of a role of the GH/IGF system in renal physiology and pathophysiology that have appeared over the past few years.

2. Expression of GH/IGF system in the rodent kidney

In addition to GH and IGFs in the circulation, locally produced IGFs and IGFBPs also affect the kidney. IGF-I is synthesized by glomerular endothelial, epithelial and mesangial cells suggesting a possible paracrine action of IGF-I in the renal glomerulus [16]. GH receptor (GHR), GH binding protein (GHBP) and all members of IGF system are expressed in the rodent kidney [8,14–21].

GHR mRNA expression in the rat kidney, as measured by in situ hybridization, was most abundant in the proximal straight tubule, with lower levels present in the medullary thick ascending limb (mTAL) [22]. GHR expression in the mTAL, which is the site of renal IGF-I synthesis, supports the view that GH has a direct effect on renal IGF-I synthesis [22]. mTAL is also the only point where both GHR and IGF-I receptor (IGF-IR) mRNAs are expressed. However, GHR mRNA is most abundant in the straight portion of the proximal tubule, where IGF-IR mRNA was not detected, suggesting direct regulation by GH in this part of the nephron. To our knowledge, no localization studies have so far been performed in mice, but Northern blot and reverse transcriptase polymerase chain reaction (RT-PCR) techniques gave evidence that GHR and GHBP mRNAs are abundantly expressed in the mouse kidney, and that their expression is altered in different experimental states accompanied by kidney growth [19,21,23–26].

Gene and protein expression of the IGF system in the kidney has been studied in detail in rats [14,15,17]. However, data on the expression of IGF system in the mouse kidney are less abundant. In a study performed in our laboratory, spatial and temporal gene expression of the IGF system during mouse nephrogenesis was assessed by non-radioactive in situ hybridization combined with quantitative RT-PCR [18] (Fig. 1). IGF-I, IGF-II and IGF-IR mRNAs are widely distributed in the developing mouse kidney. In the mature mouse kidney, IGF-I mRNA is localized in glomeruli, distal tubules, thick limb of Henle's loop and peritubular

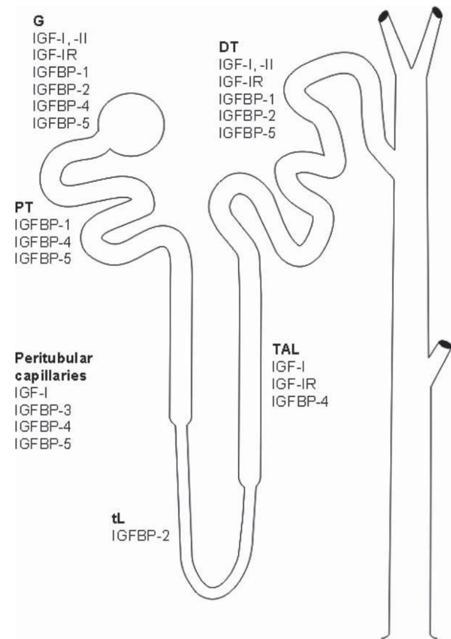

Fig. 1. IGF system gene expression in the mouse kidney [18]. G, glomerulus, PT, proximal tubule, DT, distal tubule, tL, thin limbs of the Henle's loop, TAL, thick ascending limbs of the Henle's loop.

capillaries and its levels increase after birth, indicating its role in postnatal renal physiology. IGF-II mRNA on the other hand is high in fetal kidney, reaching peak around birth and decreasing postnatally. IGF-IR mRNA expression pattern, closely matching that of rat [22], mostly overlaps with those of IGFs, in accordance with its IGF-I and -II signal transducing role [18,27]. The IGF-IR mRNA levels are highest during the initial period of metanephric development with expression sites distributed throughout the renal parenchyma and heavily concentrated at the ureteric bud and its branches, the sites of active epithelial–mesenchymal interactions leading to nephrogenesis [28]. Renal IGF-IR mRNA levels decline during subsequent stages of gestation and especially in the postnatal period.

The ability of IGF-I to regulate various cellular processes in kidney tissue depends not only on the levels of IGF-I and presence of intact IGF-IR signaling but also on local levels of IGFBPs. IGFBPs are detected in mouse kidney at different developmental stages, on both mRNA and protein levels [18,20]. IGFBP-2, -4 and -5 mRNAs are localized in differentiating cells of the mouse fetal kidney [18]. IGFBP-1 mRNA in fetal mouse kidney is concentrated in renal capsule, while in mature

kidney it is localized in glomerulus, proximal and distal tubules [18]. During nephrogenesis, IGFBP-2 mRNA expression shows similar temporal pattern as IGF-II mRNA, high prenatal levels peak around birth and decrease postnatally [18]. In the mature kidney IGFBP-2 gene is strongly expressed in the glomerulus where its protein might bind circulating and/or locally produced IGFs and facilitate their renal clearance [29] and in thin limbs of Henle's loop where IGFBP-2 might modulate IGF-II action in Na^+ reabsorbtion from the glomerular ultrafiltrate [30]. Low fetal IGFBP-3 mRNA levels increase after birth, coinciding with increased IGF-I mRNA levels [18]. Mature kidney IGFBP-3 mRNA is found exclusively in endothelial cells of the renal capillary system, which is not surprising considering its major role in carrying circulating IGF-I. In the mature kidney, IGFBP-4 mRNA is localized in mesangial cells of glomeruli, peritubular capillaries and thick limbs of Henle's loop and highest expression is found in proximal tubules [18]. In the developing mouse kidney IGFBP-5 mRNA is localized at sites of cellular differentiation [18]. Its low fetal mRNA levels increase after birth, implying its role in functional processes in the kidney. In the mature kidney, IGFBP-5 mRNA is predominantly located in glomerular mesangial cells and juxtaglomerular cells, peritubular capillaries of the medulla and distal tubules [18]. IGFBP-6 mRNA is weakly expressed in the mouse kidney, being undetectable in the adult kidney. Similar expression pattern of IGF system mRNA, mostly co-localizing with its protein, was observed in kidney of adult Snell dwarf mice [31]. Northern blot analysis revealed abundant expression of IGFBP-1 to -5, while IGFBP-6 could not be detected in the adult mouse kidney [8,19,21]. However, IGFBP-6 was detected by immunohistochemistry in epithelia of the distal tubule, and to some extent in epithelia of proximal tubule in the adult mouse kidney [32].

Taking into account the discrete spatial and temporal expression patterns of the IGFBP genes in mouse kidney, it is reasonable to assume their modulatory role of IGF action during kidney development and growth. In a number of experimental models, alterations in IGF-I expression in the kidney coincide with morphological or physiological changes of the kidney.

3. The role of the GH/IGF system in renal pathophysiology

Involvement of GH, IGFs and IGFBPs in glomerular haemodynamics, tubular function and renal development as well as their processing by the kidney in a normal setting, have been extensively reviewed previously [14,15]. Alterations in the GH/IGF axis, both locally in the kidney and in the circulation, have been linked to kidney enlargement in experimental mouse models.

Both GH and IGF-I increase renal plasma flow (RPF) and glomerular filtration rate (GFR). These effects of GH are likely mediated by IGF-I, as IGF-I affects these parameters within minutes to hours while GH effects are delayed until serum IGF-I levels rise [15,33,34]. Systemic administration of IGF-I causes increases in RPF and GFR in both human and rat [35–37] and an increase in kidney size in rats and mice [38–40]. Novel peptides called IGF displacers which bind to IGFBP but not to the IGF-IR, produce "IGF-I-like" effects including increased kidney size [41,42]. Overexpression of IGF-I in transgenic (TG) mice induces renal and glomerular hypertrophy [43–45]. Infusion of IGF-I directly into the renal parenchyma results in hypertrophy and hyperplasia of the mTAL and distal tubule whereas proximal tubules are not affected [46], along with minimal IGF-IR gene expression in proximal tubules [29]. IGF-I produced in the mTAL may play a trophic role in conditions associated with increased workload such as unilateral nephrectomy and high-protein diet.

In newborn IGF-I null mice, no renal developmental abnormalities have been reported [47,48]. In rodents, kidney development continues postnatally and 95% of IGF-I KO mice die immediately after birth. Mice that survive are smaller than wild type littermates and their kidneys have proportionally lower weight, along with reduced glomerular size and number of nephrons, suggesting that IGF-I plays a determining role for the nephron number and enhances the function in developing kidneys [49]. In two models of hepatic IGF-I deficiency kidney weight is decreased probably as a result of the decreased serum IGF-I levels since systemic delivery of IGF-I has been shown to increase kidney size [50,51].

Kidney weight is increased in IGF-II TG mice [52,53]. IGFBP-1 TG mice display a small reduction of kidney weight, although proportional to body weight [54,55], and develop glomerulosclerosis without glomerular hypertrophy [56]. IGFBP-2 overexpression inhibits GH-stimulated body and kidney growth in GH TG mice, demonstrating inhibitory effect of this IGFBP in vivo [57]. Kidney growth is disproportionately retarded also by IGFBP-3 overexpression, most likely by inhibiting IGF action although IGF-independent growth inhibitory mechanism is not excluded [58].

Renal enlargement has been shown to coincide with increase in renal extractable IGF-I levels both in rats and mice. In vitro, IGF-I is a mitogen for cultured glomerular mesangial cells and stimulates the production of extracellular matrix (ECM) by mesangial cells [34,59,60]. However, IGF-I seems not to be involved in the GH-induced glomerulosclerosis. Although both GH and IGF-I overproduction in TG mice is associated with a selective renal enlargement and glomerular hypertrophy, mesangial proliferation followed by progressive glomerulosclerosis is observed exclusively in the GH TG

mice, even though the circulating levels of IGF-I in IGF-I TG mice are greater than in GH TG mice [43,45,61]. GH could promote glomerular sclerosis directly or through the local release of growth factors other than IGF-I [62]. Components of the ECM, such as collagen types I and IV, laminin, and basement membrane heparan sulfate proteoglycan accumulate in the glomeruli of GH TG mice due to the imbalance between ECM synthesis and degradation [63–65].

The following sections will summarize recent data in support of the GH/IGF-system playing a role in the renal changes seen in diabetes, following nephrectomy and following abnormal dietary protein intake in mice, in comparison with data obtained from rat models.

3.1. Diabetes

The GH/IGF system and insulin are two major anabolic effectors and they are interlinked on many levels, promoting growth [66,67]. Well-known derangement of the GH/IGF axis in type 1 diabetes contributes to both the metabolic disturbance and susceptibility to diabetic complications, such as diabetic nephropathy, in poorly controlled diabetes [68]. The early renal changes in the diabetic disease include an increase in kidney size, glomerular volume, and GFR followed by later renal changes i.e. increased urinary albumin excretion (UAE), mesangial cell proliferation and accumulation of glomerular ECM leading to glomerular sclerosis [69] (Fig. 2(a)).

Diabetic rodents have been extensively studied as they exhibit fundamental similarities in structural and functional renal changes with diabetic patients. Kidney enlargement and haemodynamic changes seen in the insulinopenic diabetes model produced by streptozotocin (STZ), a β-cell toxin, reflect the changes seen early in the course of type 1 diabetes in humans [70,71]. Within 2 days of STZ injection, renal enlargement, increased GFR and RPF can be demonstrated [72,73]. The kidney growth in the diabetic rats is characterized both by hyperplasia and hypertrophy [74]. The non-obese diabetic (NOD) mouse, a model of spontaneous type 1 diabetes resulting from autoimmune destruction of pancreatic β-cells, is also characterised by renal hypertrophy [75].

Poorly controlled type 1 diabetes in humans is characterised by GH-hypersecretion [66], while in the previously most widely used experimental model for type 1 diabetes, the STZ-diabetic rat, serum GH levels are decreased. Recently, the mouse has been recognised as a better model for the alterations in the GH/IGF axis in diabetes, since both the STZ- and the NOD-diabetic mice are characterized by GH-hypersecretion [26,76]. With regard to the other components of the GH/IGF axis, similar changes have been reported in human and experimental diabetes in both rats and mice, with decreased circulating IGF-I and IGFBP-3 levels and increased IGFBP-1 and -2 levels reflecting changes in hepatic mRNA levels [2,68].

It has been firmly established that IGF-I accumulates in enlarged kidney of STZ-diabetic rat [77–79], STZ-diabetic mouse [76,80] and NOD mouse [25,26,75,81]. The rise in renal IGF-I that occurs in experimental diabetes paralleled with either unchanged [78,82,83] or decreased [75,80] renal IGF-I mRNA levels suggests that the accumulated renal IGF-I originates from circulation rather than from local synthesis and possibly is trapped in the kidney by IGFBPs. Pronounced changes in renal IGFBPs have been documented both in rat [84] and mouse [75,80] diabetes models.

IGFBP-1 is particularly interesting candidate both in sequestration of circulatory IGF-I and in mediating kidney growth per se. It has been proposed to act as a trans-

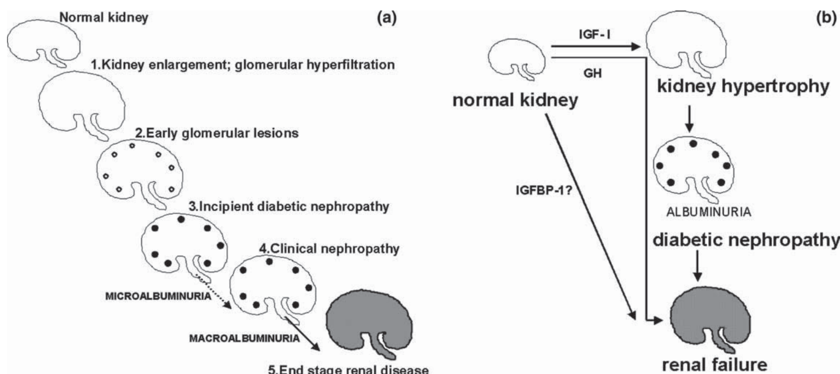

Fig. 2. A five-stage sequence for renal involvement in type 1 diabetes according to [69] (a) and a schematic illustration of the potential involvement of the GH/IGF-system in diabetic kidney disease (b).

port protein that shuttles IGFs from the circulation to the target cells [85,86]. The ability of IGFBP-1 to bind integrin receptors on cell surfaces independent of IGF-I suggests that it may also directly influence cell growth or provide a reservoir for IGF-I that could stimulate the IGF-I receptor [87]. IGFBP-1 is unique among the IGFBPs in that its serum levels are acutely dependent on food intake. Since serum IGFBP-1 levels are inversely correlated with insulin status, in type 1 diabetes circulatory levels and hepatic expression of IGFBP-1 are elevated [88,89]. Elevated levels of IGFBP-1, such as in IGFBP-1 TG mice result in features that bear similarities with the diabetic state [54,56,90] and develop glomerulosclerosis without glomerular hypertrophy [56]. Administration of a long-acting somatostatin (SS) analogue, as well as GHR antagonist (GHRA), abolish not only renal IGF-I accumulation and renal hypertrophy, but also early rise in renal IGFBP-1 mRNA in diabetic rodents [25,91].

In early STZ-induced diabetes in rats, IGFBP-1 mRNA is increased preferentially in cortical tubular cells and is strategically located to modulate IGF-I functions on kidney epithelium, such as Na^+ reapsorption, phosphate homeostasis, and gluconeogenesis [92,93]. In STZ-diabetic rats [82,93–96] and NOD mice [75,81], renal IGFBP-1 is increased at both mRNA and protein levels, whereas renal IGF-IR mRNA levels are unchanged. In another study, levels of IGF-IR and IGF-IIR mRNA have been reported to be increased in the diabetic rat kidney, accompanied by an increase in IGF-I and IGF-II binding [97]. In a recent study in the STZ-diabetic mouse model, renal IGFBP-1 concentration was unchanged whereas both IGFBP-1 mRNA and IGF-IR mRNA levels were decreased in the kidney [80]. In situ hybridization and immunostaining studies demonstrated that IGFBP-1, -3 and -5 are preferentially expressed during the rapid phase of renal growth associated with STZ-induced diabetes [93]. Northern blot analysis revealed increased IGFBP-1 and IGFBP-3 mRNA in kidney cortex of diabetic rats that displayed increased proximal tubular IGF-I binding, but not in diabetic rats that did not display this phenomenon [98]. In rats with chronic diabetes, cortical IGFBP-1 and medullary IGFBP-5 mRNAs remained elevated for 6 months [92]. Both protein and mRNA levels of IGFBP-3 and -4 are decreased in diabetic mouse kidney [75,80].

The decrease in hepatic GHR and GHBP mRNA levels, as well as liver membrane binding capacity and decreased serum IGF-I in spite of increased circulating GH in early diabetes in mice, suggest a state of GH-resistance, in agreement with data collected in rats [23,26,80,82]. While renal GHR expression in early diabetes was found to be increased in rats [82,99], renal GHR mRNA has consistently been found to be decreased in diabetic mice [23,25,26,80] with unchanged [25,26] or decreased GHBP mRNA levels [23,80].

Early rise in renal IGF-I and initial renal hypertrophy is blunted in GH-deficient diabetic dwarf rats [100–102]. Isolated GH/IGF-I deficiency also reduces the degree of renal and glomerular hypertrophy and the increase in UAE in long-term diabetes [103]. Treatment with a long-acting SS analogue, octreotide, prevents the rise in kidney IGF-I content, renal/glomerular hypertrophy and increased UAE in diabetic rats [104,105] and mice [81,106]. It was suggested that octreotide might reduce sequestering of IGF-I by IGFBP-1 in kidney by down-regulating IGFBP-1 expression, although such an effect of octreotide is in contrast to its known effects on IGFBP-1 expression in non-diabetic rats [68,91]. While GH TG mice exhibit enlarged glomeruli, mesangial matrix expansion, glomerular cell proliferation and tubulo-interstitial lesions whether diabetic or not, GHRA TG mice were protected from diabetes-induced kidney damage [44,45,61,63,107–109]. Likewise, diabetes-associated kidney damage is reduced in genetically modified mice that have a disrupted GHR gene [110]. Administration of a GHRA in diabetic mice abolished renal IGF-I accumulation, renal/glomerular hypertrophy and diminished the increase in UAE [25,76]. GHR blockade prevents alterations in the early steps of GH signal transduction, as well as diabetes-induced renal enlargement in kidneys of diabetic rodents and may represent a new concept in the treatment of diabetic kidney disease [111,112].

The knowledge we have today indicates that the GH/IGF axis may be responsible for both early and late renal changes in experimental diabetes (Fig. 2(b)). Increasing number of studies give evidence for a prominent role of number of other growth factors and cytokines in diabetic complications [2,113,114]. Recently demonstrated therapeutic effect of hepatocyte growth factor (HGF) in a STZ-rat model, associated with suppression of renal transforming growth factor β1 (TGF-β1) and mesangial connective tissue growth factor (CTGF) upregulation deserves further attention as it had effect on advanced rather than early diabetic nephropathy [115].

3.2. Nephrectomy

Both in experimental animals and man, loss of renal tissue due to a pathological process, uninephrectomy or renal ablation is followed by compensatory hypertrophy of residual nephrons, increasing the area for transport by tubular epithelia [116,117]. Hypertrophy of tubules occurring during compensatory renal growth (CRG) represents a substantial part of the increase in renal mass [118,119]. Alterations in glomerular hemodynamics associated with renal ablation, including glomerular hyperfiltration, are important components of the adaptive response but may also have adverse effects and contribute to progressive damaging of remnant glomeruli [120,121]. Unilateral nephrectomy in mice leads to compensatory enlargement of the remaining kidney associat-

ed with an increase in glomerular volume (but not number) and enlargement of the proximal convoluted tubules [122–124]. During an observation period for up to one year, no quantitative differences were detected in proteinuria or histopathology (apart from glomerular enlargement) between control mice and those with a remnant kidney [125].

Increased workload on tubules caused by hyperfiltration could lead to cell growth by mechanisms mediated by growth factors. GH and IGF-I have been implicated as pathogenic factors in CRG following unilateral nephrectomy in rodents. Hypophysectomized rats have blunted CRG in response to unilateral nephrectomy [126,127]. Furthermore, administration of long-acting SS analogue octreotide [104], GH-releasing factor antagonist [128] or a specific GHRA [123] abolishes early CRG in rodents. However, differences in relation to age and gender have been documented in response to nephrectomy. The initial phase of CRG following unilateral nephrectomy is GH-dependent in adult rats and GH-independent in immature rats [128–131]. In adult mice CRG is strictly GH-dependent [123]. Furthermore, sexual dimorphism has been demonstrated in remnant kidney growth and function two months following uninephrectomy and testosterone was suggested to be the driving force for the enhanced remnant kidney growth and glomerular hypertrophy [132]. Unlike findings in nephrectomized adult male rats, the initial phase of CRG in both adult and immature female rats is GH-independent and associated with significant hyperplasia in remnant kidneys [133].

In both rats [77,104,134–137] and mice [123,124] renal IGF-I protein content is transiently increased in the remnant kidney undergoing CRG after nephrectomy and this increase is correlated to the degree of renal ablation [138]. These changes take place in the absence of changes in hepatic or circulating IGF-I levels indicating paracrine/autocrine role for IGF-I in CRG. GHRA abolishes not only nephrectomy-induced renal and glomerular hypertrophy but also renal IGF-I accumulation without affecting circulating levels of GH and IGF-I, presumably by directly blocking kidney GHRs [123]. Whether the increase in renal IGF-I content represents changes in synthesis and/or uptake of circulating IGF-I by the kidney has not been clearly established. IGF-I mRNA levels in remnant kidney in rats have been reported to be increased [129,139] or unchanged [136], and in mice even decreased [123], making it hard to give a final conclusion. There even seems to be some differences in adult vs. immature animals [128,129]. Early CRG in the adult male rat is GH-dependent, but independent of changes in the renal IGF-I system. In contrast, CRG is GH-independent in juvenile male rat and is associated with significant increases in the renal IGF-I system. In the normal kidney, IGF-I immunoreactivity is found mainly in cells in the medullary collecting ducts and in parts of the thin limb of Henle's loop located in the outer medulla and inner medulla contains significantly higher content of IGF-I than the cortex [140,141]. During CRG, cortical IGF-I content increases as all parts of the collecting ducts, including those in the cortex and cells in the entire thin limb of Henle's loop exhibit IGF-I immunoreactivity [141]. Renal gene expression of GHR, GHBP, IGF-IR and different IGFBPs is also altered following nephrectomy but the importance of these changes has not been given much attention so far [123,129,131,133]. Early CRG in the weanling rat is associated with rapid increases in renal IGF-I mRNA followed by a rise in c-fos and c-jun gene expression and a mitogenic response [142]. Once the IGF-I mRNA levels and early response genes return to baseline levels, mitogenic growth stops and slower prolonged hypertrophic renal growth ensues. In adult rats, which exhibit predominantly hypertrophic renal growth early after nephrectomy, neither IGF-I nor early response genes mRNA levels were elevated in the kidney [74,142]. During CRG in the normal mouse, cell division and hypertrophy occur in similar proportions, while in GH-deficient mouse only cell size is markedly increased [143]. Extending these studies, the same authors suggested that with GH-deficiency growth can be achieved by cell hypertrophy and with insulin-deficiency growth can occur by cell division, whereas with combined insulin and GH-deficiency renal cell growth is suppressed as both modes of cellular growth are inhibited [144].

The predisposition to glomerulosclerosis was suggested to depend largely on the genetic background in the mouse [145]. Mutation resulting in a congenital 50% reduction in nephron number in two compared mouse strains, lead to development of glomerulosclerosis in only one of them, despite an identical reduction in nephron number and an equal increase in glomerular size [145]. Nephrons at young age have greater plasticity and compensatory growth potential than the mature nephrons, so it is not surprising that nephrectomy induces higher glomerular and renal hypertrophy in younger than in older mice [146]. When 50% reduction in nephron number was induced in a sclerosis-prone mouse strain by uninephrectomy in adult mice, sclerosis was less severe than when nephron reduction occurred in utero [147]. These findings confirmed previous reports of age-dependant response to nephrectomy in rats [104,148].

Intervention either at the GHR [123] or IGF-IR [149] level by specific antagonists, blunts or abolishes renal hypertrophy following uninephrectomy, giving direct evidence for involvement of GH/IGF system in the CRG. However, different growth factors might have a role in different stages of CRG after nephrectomy. Recently it was shown that CRG is vascular endothelial growth factor (VEGF)-dependent as in VEGF-Ab-treated uninephrectomized mice, increased glomerular volume was abolished and renal hypertrophy was blunted

[124]. Interestingly, the renal effects of VEGF-Ab administration occurred without affecting the transient renal IGF-I accumulation, indicating VEGF to be a downstream mediator of IGF-I [124,150].

3.3. Dietary protein intake

Nutrition (i.e., dietary protein and/or energy intake) is one of the main regulators of the GH/IGF system [151]. Dietary protein content has profound effects on renal metabolic activity, size and morphology in both the healthy and diseased kidney [14,152–154].

In aging rats with intact kidneys, HP-diet increases UAE and worsens renal lesions compared to animals on standard-protein (SP) diet, while kidneys of low-protein (LP)-fed animals are almost free of lesions [155]. Rats chronically fed LP display reduced RPF and GFR, when compared to rats fed a HP-diet [156]. Kidney weight increases linearly with protein intake rate both in male and female mice, whereas GFR is significantly elevated only in male mice fed HP diet [154].

Renal IGF-I levels are elevated in HP-fed rats, particularly in glomeruli and suppressed in the LP-fed rats [157,158]. Persistent elevation of circulating and/or renal IGF-I levels does not appear necessary for the maintenance of kidney hypertrophy in the HP-fed rat, but seems to play a role in the initiation of the process [158]. A number of IGFBPs are synthesized in the rodent kidney [17,18] and their changed expression in response to protein intake can play a role in modulating IGF-I actions in the kidney [21,159,160]. However, changes in renal expression of IGFBP-1 deserve particular attention since it is co-localized with IGF-I at both the mRNA [29] and protein level [140] in the mTAL, a nephron segment particularly undergoing hypertrophy in response to HP-diet [161,162]. Furthermore, their gene expression in this segment is inversely regulated by GH, with IGF-I increasing as IGFBP-1 decreases in response to GH [29]. Rats switched to HP diets demonstrated increased IGF-I and decreased IGFBP-1 mRNA levels in mTALs, whereas those switched to LP showed inverse changes [162,163]. Similarly, renal IGF-I mRNA levels are reduced and IGFBP-1 mRNA increased in response to short-term fasting [164,165]. These fasting- and HP-induced renal changes take place both in GH-deficient dwarf rats as well as in normal rats, suggesting that the effects of dietary protein are not mediated by GH [160,162]. HP-induced increase in kidney weight and renal IGF-I accumulation was not affected by GHRA administration in mice, confirming the process to be indeed GH-independent [21]. Although dietary protein may regulate GH secretion, it has been established that the acute effects of GH on RPF and GFR increase are mediated by IGF-I [33]. Thus GH is probably not a mediator of the acute increase in renal hemodynamics induced by a protein load and it is possible that the rise in IGF-I contributes to the increase in renal hemodynamics that occurs with a HP diet.

Experimental and clinical evidence indicate that HP-diet accelerates the progression of kidney lesions and functional deterioration in chronic renal failure (CRF) [166,167]. On the contrary, dietary protein restriction has been associated with both amelioration of renal function and attenuation of morphologic signs of renal injury [155,168–170] along with changes at the molecular level in mesangial matrix in various models of experimental renal disease [171–173]. Occurrence of CRG after nephrectomy is significantly blunted in rats maintained on LP diet but enhanced in animals fed HP [174]. In diabetic rats, LP-diet significantly reduced GFR and RPF as well as features of diabetic glomerulopathy including mesangial hypercellularity and mesangial matrix expansion [168]. In experimental animals with renal ablation or experimentally induced diabetes, increases in glomerular pressures and flows precede proteinuria and glomerular sclerosis. Protein restriction prevents these hemodynamic adaptations as well as the late complications [175].

Blunting of CRG after nephrectomy caused by LP diet is accompanied by a failure to increase kidney IGF-I levels to those seen during CRG in rats on SP- or HP-intake [135]. The renotrophic effect of a HP diet on CRG seems GH-dependent and IGF-I-mediated as CRG and renal IGF-I remain unaffected by the HP-intake in dwarf rats [174]. The influence of the HP-intake on the degree of CRG in nephrectomized rats has been associated with the variations in the levels of circulating IGF-I, since the HP-induced renal growth has been shown to be unrelated to the kidney IGF-I content [176]. However, in the experimental setting the possibility of such relation existing at an earlier time point in the course of CRG cannot be ruled out. In nephrectomized rats fed HP, parathyroidectomy abolished the CRG and the increase in IGF-I serum level suggesting that calciotropic factors affect the production of IGF-I at the hepatic level in response to variations in the protein intake and directly or indirectly modulate the magnitude of the CRG in response to nephron reduction [176].

In GH TG mice with established mild glomerular lesions [61,107], LP intake not only attenuated renal and glomerular hypertrophy, the degree of glomerulosclerosis and UAE, but also reduced renal expression of collagen type IV and tenascin [177]. These renal changes were accompanied by decrease in serum IGF-I levels in LP-fed GH TG mice [177]. It is of importance that protein restriction had beneficial effect although it was initiated after mice had already established glomerular disease.

Several mechanisms have been proposed to be involved in renal effects of HP diet, including nitric oxide (NO) [173,178] and prostaglandins [179,180]. Apart from GH/IGF system, various other growth factors have been documented to be involved in dietary protein

intake influence on the kidney structure and function. Dietary protein restriction [171], as well as specifically L-Arg restricted diet [173], prevent the elevated expression of TGF-β1 mRNA and protein that occurs within glomeruli after induction of experimental glomerulonephritis, abolishing an increase in ECM deposition and attenuating proteinuria. Glomerular platelet derived growth factor A (PDGF-A) and -B chain and TGF-β mRNA expression, together with histopathologic changes in experimental model characterized by focal glomerulosclerosis are all ameliorated by LP-diet [170]. Recently it was shown that glomerular hypertrophy caused by HP-diet is VEGF-dependent [181]. The administration of a neutralizing VEGF-Ab in mice fed a HP diet for one week prevented the glomerular hypertrophy, whereas HP-induced increase in renal extractable IGF-I protein and kidney weight were not affected [181].

3.4. Conclusion and perspectives

All members of GH/IGF axis are expressed in mouse kidney and exert multiple effects on renal development, growth and pathophysiology. GH and IGF-I, together with other growth factors and cytokines, have various biological effects including the modulation of cellular proliferation, motility, contractility and synthesis of ECM proteins and are important players in adaptive morphological and functional changes taking place in the kidney. It is likely that the overall net biological action of IGF-I in the kidney depends on the relative abundance of each IGFBP in the glomerular and tubulointerstitial compartments that may interact either in a synergistic or opposing manner to modulate IGF-I activity. Locally produced IGFBPs may retain IGF-I at the site of synthesis or 'extract' it from the circulation providing a local IGF-I reservoir. Factors such as cell-surface binding, matrix association, proteolysis, or phosphorylation that alter IGFBP affinity for IGFs are likely to modulate target cell actions. Studies of TG and KO mice have provided valuable information on the role of GH/IGF system in the regulation of growth and development. In the existing mouse strains, more detailed studies of regulation of this system in kidney and consequences of disruption are expected to give more definite answers. The ever-developing technology of genetic manipulation offers means for more specific models in which gene function could be ablated conditionally, in specific organs, tissues, or at specific developmental points. A causal link between changes in GH, IGF-I and different forms of accelerated kidney growth could be further addressed in kidney-specific GH/IGF axis TG and KO mice. In view of the fact that various growth factors – beside GH and IGF – may be interacting to promote the deterioration of renal function and structure, combination therapy addressing different points of action may present a new concept in the treatment of renal disease.

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Chapter 3

Kidney growth in normal and diabetic mice is not affected by human insulin-like growth factor binding protein-1 administration

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submitted

Kidney growth in normal and diabetic mice is not affected by human insulin-like growth factor binding protein-1 administration

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ABSTRACT

Background. Insulin-like growth factor I (IGF-I) accumulates in the kidney following the onset of diabetes, initiating diabetic renal hypertrophy. Increased renal IGF-I protein content, not reflected in mRNA levels, suggests that renal IGF-I accumulation is due to sequestration of circulating IGF-I rather than local synthesis. It has been suggested that IGF-I is trapped in the kidney by IGF binding protein 1 (IGFBP-1).

Methods. We administered purified human (h)IGFBP-1 to non-diabetic and diabetic mice as 3 daily sc injections for 14 days, starting 6 days after induction of streptozotocin (STZ)-diabetes when the animals were overtly diabetic.

Results. Markers of early diabetic renal changes, i.e. increased kidney weight, glomerular volume and albuminuria coincided with accumulation of renal IGF-I despite decreased mRNA levels in 20 days diabetic mice. hIGFBP-1 administration had no effect on increased kidney weight or albuminuria in early diabetes, although it abolished renal IGF-I accumulation and glomerular hypertrophy in diabetic mice. Increased IGF-I levels in kidney of normal mice receiving hIGFBP-1, were not reflected on kidney parameters.

Conclusion. IGFBP-1 administration in diabetic mice had only minor effects on diabetic renal changes. Accordingly these results are not in support of the hypothesis that IGFBP-1 plays a major role for the early renal changes in diabetes.

Keywords: diabetes, growth hormone, insulin-like growth factor I, insulin-like growth factor binding protein 1, mouse

INTRODUCTION

Long-term diabetes is accompanied by diabetic nephropathy in 15-20 % of patients with type 1 diabetes and diabetic nephropathy is one of the most frequent causes of end-stage renal failure in developed countries (1). Increased kidney size, glomerular volume and hyperfiltration are followed

by an increase in urinary albumin excretion (UAE), mesangial proliferation and accumulation of extracellular matrix. Progressive glomerular sclerosis leads to proteinuria and progressive renal insufficiency. The involvement of the growth hormone (GH)/ insulin-like growth factor (IGF) axis in diabetic renal complications has been recognized for a long time (2).

IGF-I is a multifunctional polypeptide produced under the influence of pituitary GH (3). The liver produces most of the circulating IGF-I although physiologically important autocrine and paracrine production occurs within other tissues. The biological functions of IGF-I are mediated by cell surface receptors, particularly by the IGF-I receptor (IGF-IR, also known as the Type 1 IGF receptor). Six high-affinity binding proteins (IGFBP-1 to -6) modulate the actions of IGF-I (4). Circulatory IGFBPs carry the major portion of serum IGF-I, neutralizing its hypoglycemic potential, prolonging its half-life and transporting it to target tissues. Locally synthesized IGFBPs modulate biological effects of IGF-I on the receptor level. In addition, IGFBPs may also have ligand-independent effects (4).

Morphological and haemodynamic renal changes that occur in the insulinopenic diabetes model produced by streptozotocin (STZ), reflect the changes seen in the course of type 1 diabetes in humans. Following the onset of diabetes, IGF-I accumulates in the kidney, preceding the initiation of renal hypertrophy (5-7). An increased kidney IGF-I protein content, along with unchanged or decreased IGF-I mRNA levels, suggest that renal IGF-I accumulation is due to sequestration of circulatory IGF-I rather than to local synthesis (6).

IGF-I may get trapped in the kidney as a consequence of changes in renal IGFBPs. IGFBP-1 is considered a strong candidate both in captivating IGF-I locally and in mediating kidney growth *per se*. IGF-I and IGFBP-1 co-localize in the same portions of the nephron, in the same cell types, supporting the view that the actions of IGF-I may be modified by IGFBP-1 (8). In early STZ-induced diabetes in rats, cortical IGFBP-1 mRNA is increased (9).

Recently, we reported increased kidney weights after human (h)IGFBP-1 administration in pituitary deficient Snell dwarf mice. In addition, hIGFBP-1 co-administered with IGF-I neutralized the stimulating effects of IGF-I on body length and weight, but not on kidney weight (10). These results, suggesting an important role for IGFBP-1 in kidney enlargement, led us to investigate the effects of hIGFBP-1 administration on kidney parameters and regulation of the GH/IGF axis in a diabetic mouse model.

MATERIALS AND METHODS

hIGFBP-1 isolation and purification

hIGFBP-1 was isolated from midgestational amniotic fluid obtained for diagnostic purposes with approval of the Ethics Committee (Sophia Children's Hospital, Rotterdam, the Netherlands) and purified on a RESOURCE®RPC 3 ml column connected to a FPLC® System (Automatic FPLC, Pharmacia Biotech, Uppsala, Sweden) as described in detail previously (11).

Animals and procedures

Female NMRI mice (Bomholtgaard, Ry, Denmark) with initial body weight 21.3 ± 0.9 g were used. Mice were housed 5–6 per cage and had free access to standard chow (Altromin #1324; Lage, Germany) and tap water. They were kept at constant temperature (22±2°C) and humidity (55±2 %), under a 12-hours light (06.00-18.00), 12-hours dark cycle. The study complied with Danish regulations for handling laboratory animals.

The animals were randomized into 4 groups, two of which were made diabetic: (1) control/placebo, CP (n=10); (2) control/hIGFBP-1, CI (n=10); (3) diabetic/placebo, DP (n=15); (4) diabetic/hIGFBP-1, DI (n=15). After 12 hours of food deprivation (all groups), diabetes was induced at day 0 by a single i.v. injection of STZ (≈225mg/kg BW) dissolved in 0.154 M NaCl (pH=4). The animals were weighed and glucose levels in blood (Precision Xtra™, Abbott Laboratories, MediSense Products, Bedford, MA, USA) and glucose and ketone bodies levels in urine (Neostix-4, Ames, Stoke Poges, Slough, UK) were monitored daily throughout the study. Mice with ketonuria on day 3 were treated with sustained release insulin implants (Linbit™ for mice, Linshin Canada, Inc., Scarborough, Ontario, Canada) to correct ketonuria and prevent massive body weight loss. Mice with blood glucose levels above 16 mM and without ketonuria were included in the study.

hIGFBP-1 administration was initiated at day 6, when the animals developed overt diabetes. hIGFBP-1 was administered by 3 daily sc injections (8 am, 4 pm, 12 pm) for 14 days (0.3 mg hIGFBP-1/animal/day, based on previous study (10)), while placebo groups received an equivalent volume of vehicle alone (0.154 M NaCl) in the same time intervals (days 6-19). Food consumption was measured per cage and expressed per mouse. Animals were sacrificed at day 20 after sodium barbital (10 mg/kg body weight ip) anesthesia, exactly 2 hours after the last hIGFBP-1 injection. Blood was drawn five minutes later from the retro-orbital venous plexus. Serum was stored at –80°C until analysis. Immediately after extraction kidneys and liver were weighed, snap frozen in liquid nitrogen and stored at –80°C. The middle part of the left kidney (2 mm thick), containing the papilla was fixed and embedded in paraffin for morphological measurements and immunohistochemistry (see below).

Immunoassays

Serum GH, serum IGF-I and kidney IGF-I were measured by radioimmunoassay (RIA) as described previously (12). Serum insulin was measured by an ultrasensitive rat insulin enzyme-linked immunosorbent assay (DRG Diagnostics, Marburg, Germany). Semilog linearity of mouse serum and rat insulin was found at multiple dilutions, indicating antigen similarity between mouse and rat insulin. The intra- and inter-assay coefficients of variation were <5 % and <10 %, respectively.

Western ligand blotting (WLB) for determination of serum and kidney IGFBPs

SDS-PAGE and WLB analysis of serum and kidney IGFBPs were executed as described previously (13). The relative densities of the bands were expressed in pixels.

Western immuno blotting (WIB) for IGFBP-1 and IGFBP-2

Serum samples (2 µl per lane) were separated by SDS-PAGE (15 % polyacrylamide) and transferred by electroelution onto nitrocellulose membrane (Schleicher & Schuell, Munich, Germany). The blots were blocked for 12 hours at 4°C in 5 % non-fat milk and incubated overnight at 4°C with a polyclonal goat IGFBP-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dilution of 1/1000, or a polyclonal goat IGFBP-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dilution of 1/1000. The IGFBP-1 Ab cross-reacts both with human and mouse (m) IGFBP-1. After washing in TBS-Tween (0.1 %), the membranes were incubated with an anti-goat IgG Ab conjugated with horseradish peroxidase (Pierce, Rockford, IL, USA) for 1 hr at room temperature in a dilution of 1/12000. The bands were visualized by chemiluminescence (BioWest™, UPV Inc., Upland CA, USA) and quantified by a UPV BioImaging System (UPV Inc., Upland CA, USA).

Immunohistochemistry

The middle part of the left kidney (2 mm thick), containing the papilla, was fixed in neutral buffered 4 % paraformaldehyde and embedded in paraffin. 4 µm thick sections were prepared by standard techniques and mounted on SuperFrost Plus slides (Fisher Scientific). Tissue sections were then deparaffinized and rehydrated. Kidney sections were incubated with a polyclonal antibody against human IGF-I (a kind gift from Dr. Peter Gluckman, Liggins Research Institute, Auckland, New Zealand) (14) for 24 h at 4°C. The immunoreactivity was visualized by the avidin-biotin-peroxidase technique, using diaminobenzidine as the chromagen (Vectastain, Vector Laboratories, Burlingame, CA). The tissue sections were then counterstained with Carazzi's hematoxylin, dehydrated and mounted with Permount® (Fisher Scientific).

Real-time PCR

Total RNA was extracted from kidney and liver samples by TRIZOL®Reagent following the instructions of the manufacturer (GibcoBRL®, Life Technologies™, Inc. Gaithersburg, MD, USA). Renal and hepatic GH/IGF system gene expression was analyzed by means of Real-time quantitative PCR (TaqMan), as described previously (11). Following cDNA synthesis, mRNA levels were quantitatively determined on an ABI Prism® 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) using SYBR-green technology. The primers used for mouse hypoxanthine phosphoribosyltransferase (mHPRT), mIGF-I, mIGFBP-1, mIGFBP-3, mGH-receptor (GHR) and mGHBP have been reported previously (11). The primerset used for mIGFBP-2 were: forward primer, CGCGGGTACCTGTGAAAAGA; reverse primer, CCCTCAGAGTGGTCGTCATCA, resulting in an amplicon size of 77 base pairs. The primerset used for mIGFBP-4 were: forward primer, CATCCCCATTCCAAACTGTGA; reverse primer, ACCCCTGTCTTCCGATCCA, resulting in an amplicon size of 110 base pairs. The primerset used for mIGFBP-5 were: forward primer, AGACCTACTCCCCCAAGGTCTT; reverse primer, TTTCTGCGGTCCTTCTTCACA, resulting in an amplicon size of 84 base pairs. The primerset

used for mIGF-IR were: forward primer, GGGATTTCTGCGCCAACA; reverse primer, CGTGGATGACGAAGCCATCT, resulting in an amplicon size of 62 base pairs.

PCR efficiencies for all primer sets were > 95 %. All measured values were normalised to mHPRT gene expression.

Estimation of glomerular volume

The middle part of the left kidney (2 mm thick), containing the papilla, was fixed in 2 % paraformaldehyde, 2.5 % glutaraldehyde, 0.1 M cacodylate buffer and embedded in paraffin. Sections (2 µm) cut on a rotation microtome (Leica Rotation Microtome RM 2165, Leica, Vienna, Austria) were stained with periodic acid-Schiff and hematoxylin. The thickness of the sections was controlled routinely by a Digital Microcator ND 221 (Heidenhain, Traunreut, Germany) attached to the microscope. In each animal, the mean glomerular tuft volume (V_G) was determined from the mean glomerular cross-sectional area (A_G) by light microscopy at a magnification of 40x as previously described (12). Profile areas were determined with a 2D-version of the nucleator (CAST, Olympus, Denmark). A_G was determined as the average area of a total of 40 to 50 glomerular profiles (capillary tuft omitting the proximal tubular tissue within the Bowmann capsule) and V_G was calculated from following equation: $V_G = \beta/k \times (A_G)^{3/2}$, where $\beta = 1.38$ is the shape coefficient for spheres (the idealized shape of glomeruli), and $k = 1.1$ is a size distribution coefficient. The measurements were performed in a blinded fashion without the investigator's knowledge of the different groups.

Urinary albumin and creatinine concentration

The urine samples were collected in the morning (8-11 am) by gentle massage over the lower part of the abdomen and stored at –20°C until analysis was performed. The urinary albumin concentration was determined by RIA as previously described (12). The urinary creatinine concentration was measured by an automated technique adapted from the method of Jaffé and corrected for the prevailing glucose content due to interference in the Jaffé reaction. The intra- and inter-assay coefficients of variation were <5 % and <10 % respectively for both assays.

Statistical analysis

Means were compared by one-way ANOVA followed by pairwise comparisons with the least significant difference (LSD) method. Serum GH values <1.3 were assigned value 1.3 and 4 groups were tested by Kruskal-Wallis test, followed by pairwise comparisons by Mann-Whitney test (SPSS software package). Results are given as mean ± SEM, with n indicating the number of animals studied. Significance was assigned at $P \leq 0.05$.

RESULTS

Body weight and metabolic parameters

Body weights of mice from control groups steadily increased over the time of the study (Fig.1a). Diabetic mice weighed significantly less than their respective control groups at all time points considered throughout the study, despite a significantly higher daily food consumption (Fig.1c). At day 2, blood glucose levels in diabetic animals were approximately 3-fold higher than in controls and remained elevated throughout the study period (Fig.1b). Insulin levels at day 20 were lower in both diabetic groups compared to their respective controls (Fig.2c). Injected hIGFBP-1 did not have any influence on mentioned parameters in the control and diabetic groups. Blood glucose levels were significantly decreased in diabetic mice administered hIGFBP-1 only in the last time point measured (P=0.016).

Serum GH, IGF-I and IGFBPs

In diabetic animals, serum GH levels were significantly increased (Fig.2a) and IGF-I levels were decreased (Fig.2b). While hIGFBP-1 had no additional effect on GH serum levels, it further decreased IGF-I serum levels (Fig. 2a, 2b).

Serum levels of IGFBP-1, detected by WLB, were markedly higher in animals injected with hIGFBP-1, but significantly less elevated in the diabetic than in the control group (Fig.2d). On WIB, injected hIGFBP-1 and endogenous mIGFBP-1 were clearly distinguished by size (Fig. 2e). mIGFBP-1 levels were not different between the groups. Circulating IGFBP-2 levels, detected both on WLB and WIB, were significantly elevated in diabetic animals receiving hIGFBP-1 compared to all other groups (Fig.2d, 2e). Serum IGFBP-3 values were lower in diabetic animals compared to their respective controls and significantly reduced by hIGFBP-1 injections in both control and diabetic groups (Fig.2d). Serum IGFBP-4 levels were decreased in both diabetic groups and significantly increased in normal mice injected with hIGFBP-1 (Fig.2d).

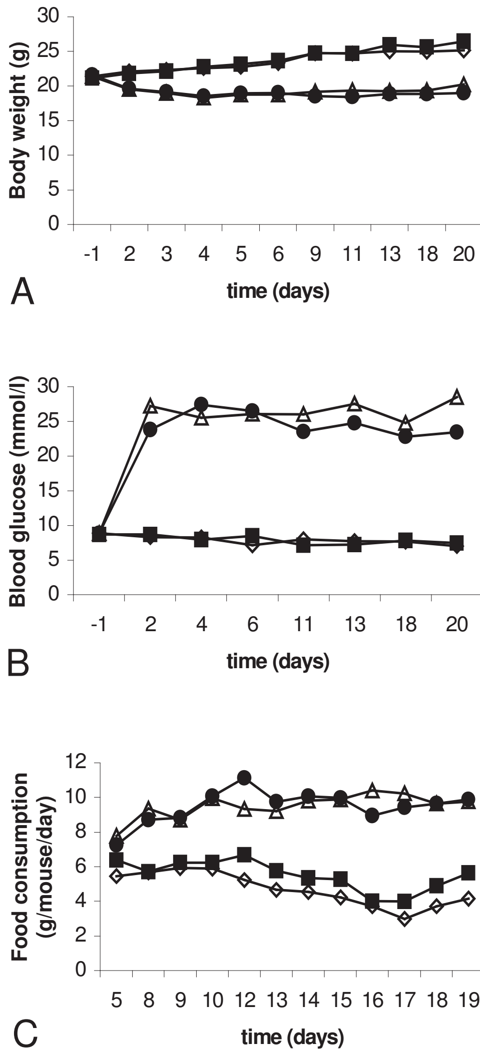


Figure 1
Body weight (A), blood glucose (B) and food consumption (C) in non-diabetic mice injected with placebo (white diamond) or hIGFBP-1 (black square) and diabetic mice injected with placebo (white triangle) or hIGFBP-1 (black circle). Values are given as means (n=10 for control, n=15 for diabetic groups).

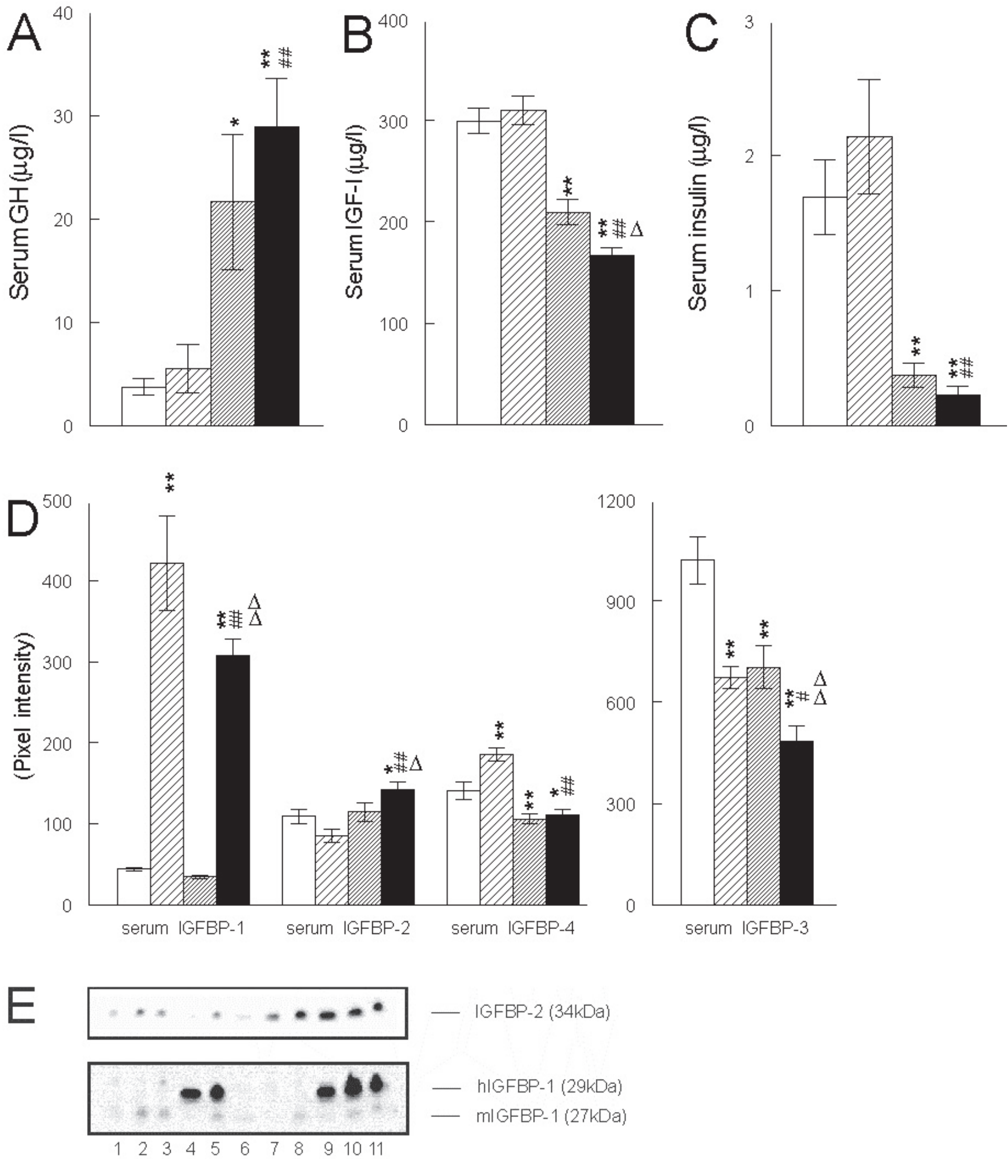


Figure 2
Serum GH (A), IGF-I (B), insulin (C) and IGFBPs (WLB) (D) levels at day 20 in non-diabetic mice injected with placebo (CP, white bars) or hIGFBP-1 (CI, light shaded bars) and diabetic mice injected with placebo (DP, dark shaded bars) or hIGFBP-1 (DI, black bars). Values are means ± SEM (n=10 for control, n=15 for diabetic groups). $^{**}P \leq 0.005$, $^{*}P \leq 0.05$ vs. CP group, $^{\#\#}P \leq 0.005$, $^{\#}P \leq 0.05$ vs. CI group, $^{\Delta\Delta}P \leq 0.005$, $^{\Delta}P \leq 0.05$ vs. DP group. Serum IGFBP-1 (hIGFBP-1, 29 kDa; mIGFBP-1, 27 kDa) and IGFBP–2 (34 kDa) by WIB in CP (lane 1-3), CI (lane 4,5), DP (lane 6-8) and DI (lane 9-11) groups (E)

Kidney weight, glomerular volume and urinary albumin excretion

Kidney weight is represented as the mean value of both kidneys (Fig.3a). Kidneys of diabetic mice were significantly heavier than kidneys of their respective non-diabetic controls. Administration of hIGFBP-1, both in the control and diabetic groups, did not have any effect on kidney weight.

Glomerular volume was increased as a consequence of the diabetic state and injection of hIGFBP-1 decreased it significantly (Fig.3b). As an indicator of proteinuria the albumin/creatinine ratio (mg/mmol) was calculated. This ratio was increased in diabetic mice (Fig.3c). The effect of hIGFBP-1 was not statistically significant.

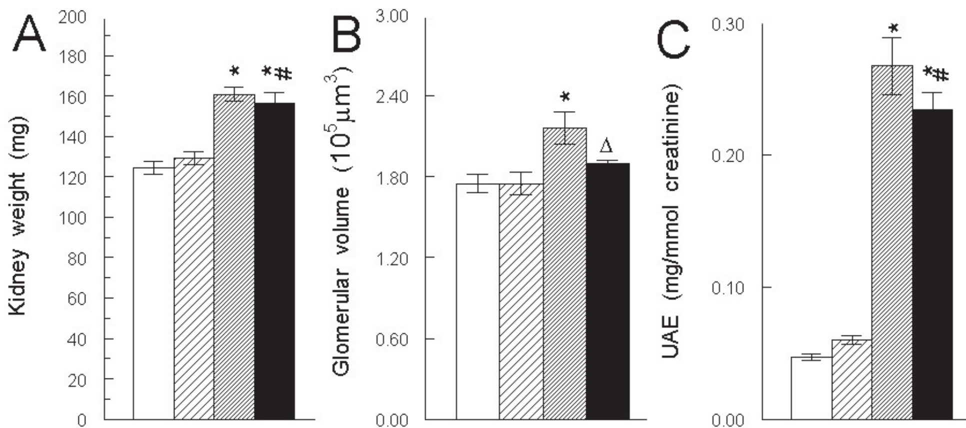


Figure 3
Kidney weight (A), glomerular volume (B) and urinary albumin excretion (C) at day 20 in non-diabetic mice injected with placebo (CP, white bars) or hIGFBP-1 (CI, light shaded bars) and diabetic mice injected with placebo (DP, dark shaded bars) or hIGFBP-1 (DI, black bars). Values are means ± SEM (n=10 for control, n=15 for diabetic groups). $^*P \leq 0.005$ vs. CP group, $^{\#}P \leq 0.001$ vs. CI group, $^{\Delta}P \leq 0.05$ vs. DP group

Kidney IGF-I and IGFBPs

Extractable IGF-I protein content was increased in kidney of diabetic mice receiving placebo (Fig.4a). In diabetic mice receiving hIGFBP-1, IGF-I protein content was reduced (Fig.4a). hIGFBP-1 administration increased IGF-I levels in kidney of normal mice (Fig.4a). Immunostainable IGF-I was clearly visible in both diabetic groups, but was absent in both non-diabetic groups. In diabetic kidneys, IGF-I was predominantly localised in papillary collecting ducts (Fig. 4c) and to some extent in cortical collecting ducts.

Renal IGFBP-1 protein levels were markedly increased in groups receiving hIGFBP-1 and they were higher in diabetic than control group (Table 1 and Fig.4b). IGFBP-2 protein content was higher in kidneys of diabetic animals receiving hIGFBP-1 compared to all groups (Table 1). Kidney IGFBP-3 protein levels were decreased in both diabetic groups (Table 1). The renal IGFBP-4 protein level was decreased in diabetic mice receiving placebo with an increase due to hIGFBP-1 administration (Table 1).

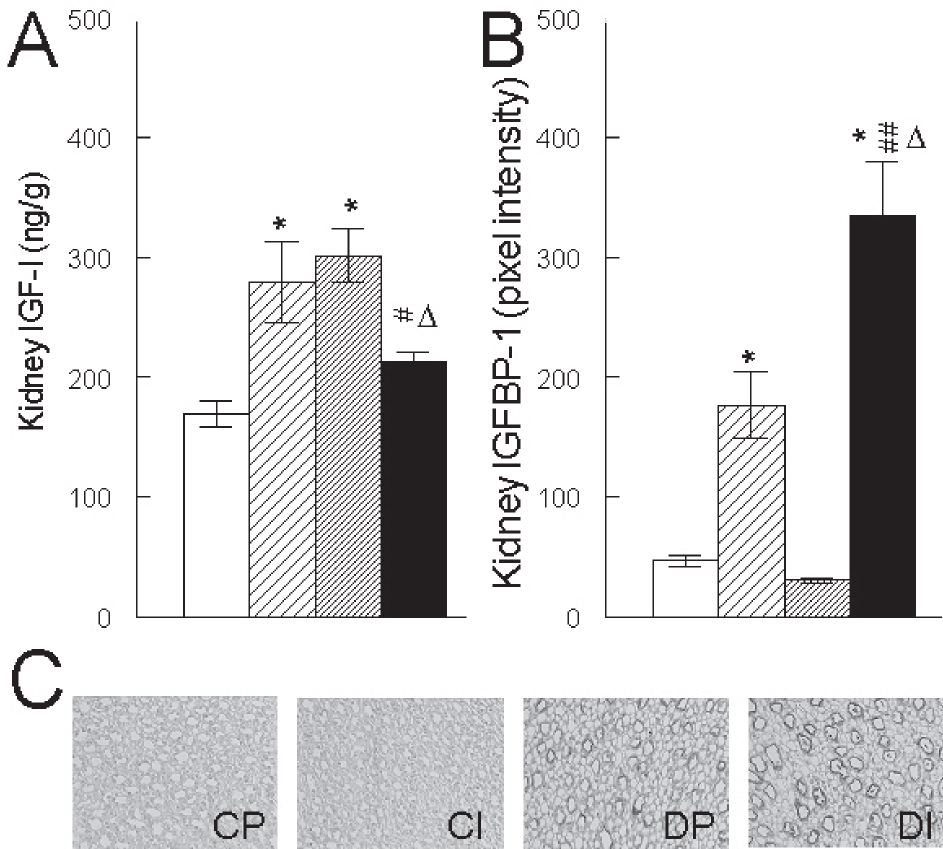


Figure 4
Kidney IGF-I (A) and IGFBP-1 (B) levels and immunostainable IGF-I in papillary collecting ducts (20X) (C) at day 20 in non-diabetic mice injected with placebo (CP, white bars) or hIGFBP-1 (CI, light shaded bars) and diabetic mice injected with placebo (DP, dark shaded bars) or hIGFBP-1 (DI, black bars). Values are means ± SEM (n=10 for control, n=15 for diabetic groups (A); n=9 (B)). $^{*}P \le 0.005$ vs. CP group, $^{\#\#}P \le 0.005$, $^{\#}P \le 0.05$ vs. CI group, $^{\Delta}P \le 0.001$ vs. DP group

Table 1. Kidney IGFBPs mRNA (artificial values, CP value set as 1) and protein levels (pixel intensity) at day 20 in non-diabetic mice injected with placebo (CP) or hIGFBP-1 (CI) and diabetic mice injected with placebo (DP) or hIGFBP-1 (DI). Values are means ± SEM (n=10 (mRNA); n=9 (protein)). *$P \leq 0.05$ vs. CP group, $^{\#}P \leq 0.05$, vs. CI group, $^{\Delta}P \leq 0.05$ vs. DP group

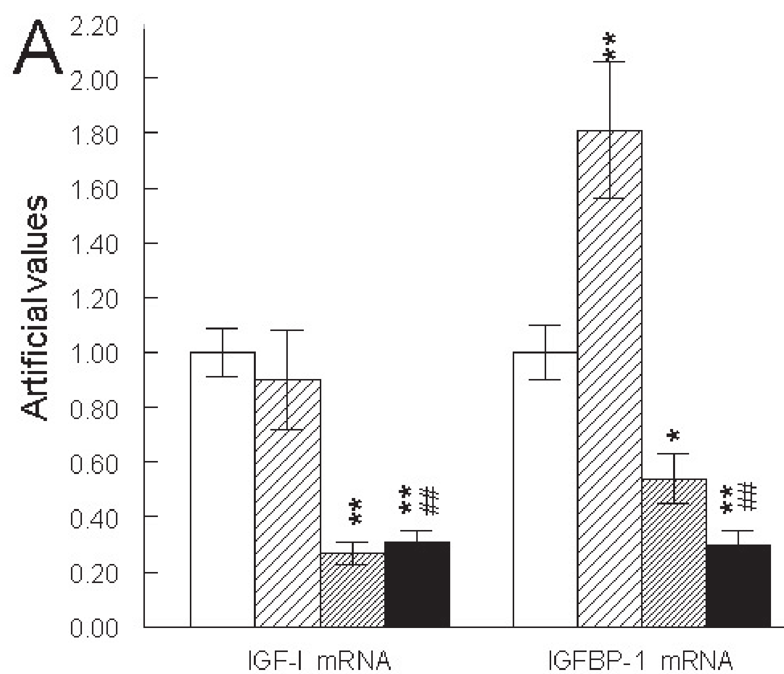
Kidney	mRNA				protein			
	CP	CI	DP	DI	CP	CI	DP	DI
IGFBP-1	1±0.11	1.81±0.25*	0.54±0.09*	0.3±0.05#	46.8±4.8	176.5±27.5*	30.6±2.3	334.4±45.6#,Δ
IGFBP-2	1±0.15	0.86±0.14	0.73±0.09	0.81±0.12	357.8±20.2	358±22	334.4±22	485.9±29#,Δ
IGFBP-3	1±0.05	1.04±0.1	0.47±0.02*	0.4±0.04#	331.8±28.5	318.5±14.6	197.9±14.9*	189.7±8#
IGFBP-4	1±0.09	0.9±0.08	0.46±0.06*	0.41±0.04#	160±12	136.5±13.5	79.5±3*	113.9±9.7Δ
IGFBP-5	1±0.06	0.85±0.12	0.65±0.05*	0.6±0.03#				

Expression of the GH/IGF axis genes in kidney and liver

Kidney IGF-I mRNA concentration was significantly reduced in both diabetic groups (Fig.5a). IGFBP-1 mRNA levels were increased in the control group receiving hIGFBP-1 and decreased in both diabetic groups (Table 1 and Fig.5a). Expression of IGFBP-2 mRNA in kidney was not significantly different between the groups (Table 1). Renal mRNA expression of IGFBP-3 to –5 was decreased in both diabetic groups (Table 1). Levels of renal IGF-IR mRNA were significantly lower in all groups compared to control/placebo group ($P<0.001$, data not shown). Expression of GHR mRNA was decreased in the control group receiving hIGFBP-1 ($P=0.018$, CI vs. CP) and diabetic group receiving placebo ($P=0.014$, DP vs. CP, data not shown). GHBP mRNA levels were decreased in kidneys of both diabetic groups ($P\leq0.001$, DP vs. CP and DI vs. CI, data not shown).

Hepatic IGF-I mRNA was downregulated in diabetes and upregulated in normal mice receiving hIGFBP-1 (Fig.5b). Liver IGFBP-1 mRNA levels were significantly increased in diabetic animals receiving hIGFBP-1 compared to all groups (Fig.5b). IGFBP-2 mRNA concentration was elevated in diabetic livers ($P<0.001$, DP vs. CP; $P=0.007$, DI vs. CI, data not shown). Hepatic IGFBP-3 mRNA was decreased in diabetic mice ($P=0.001$, DP vs. CP) and restored to control levels when hIGFBP-1 was injected ($P=0.013$, DI vs. DP, data not shown). Levels of IGFBP-4 and –5 mRNA were not different between the groups (data not shown). Expression of GHR and GHBP mRNA was decreased in livers of both diabetic groups ($P\leq0.001$, DP vs. CP and DI vs. CI, data not shown).

Figure 5
Expression of IGF-I and IGFBP-1 mRNA in kidney (A) and liver (B) at day 20 in non-diabetic mice injected with placebo (CP, white bars) or hIGFBP-1 (CI, light shaded bars) and diabetic mice injected with placebo (DP, dark shaded bars) or hIGFBP-1 (DI, black bars). Values are means ± SEM (n=10). $^{**}P\leq0.005$, $^{*}P\leq0.05$ vs. CP group, $^{\#\#}P\leq0.005$ vs. CI group, $^{\Delta}P\leq0.05$ vs. DP group


A
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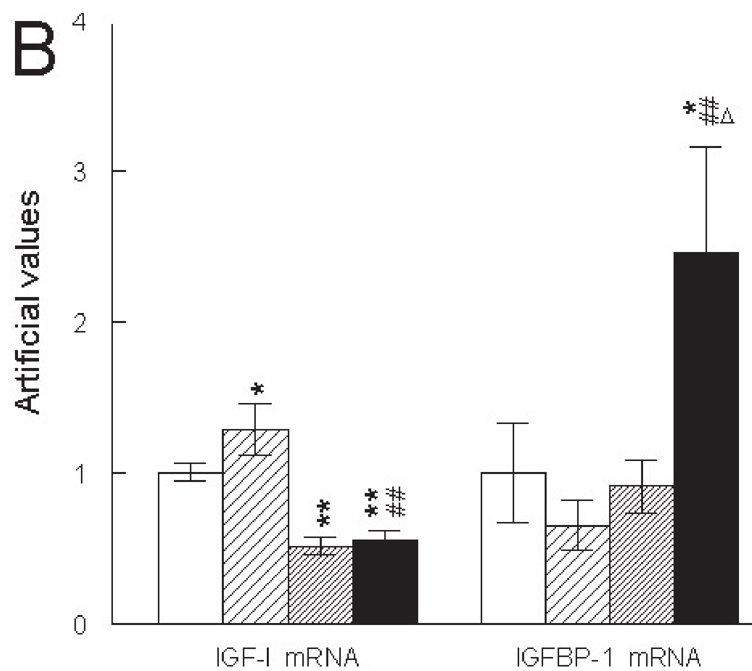
Artificial values
IGF-I mRNA
IGFBP-1 mRNA


B
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Artificial values
IGF-I mRNA
IGFBP-1 mRNA

DISCUSSION

The major new finding of this study is that hIGFBP-1 administered for 2 weeks to mice with early STZ-diabetes, had no further effect on the increase in kidney weight and albuminuria. However, hIGFBP-1 administration abolished the renal IGF-I accumulation and the increase in glomerular volume in diabetic animals.

The majority of previously published data on the involvement of the IGF system in diabetic renal hypertrophy have been obtained in the STZ-diabetic rat model, which is characterized by GH hyposecretion (2). In contrast, as recently described, the STZ-diabetic mouse model is characterized by GH hypersecretion, thereby being a better model of the changes seen in type 1 diabetes in humans (12). It has been established for a long time that IGF-I accumulates in the hypertrophying diabetic kidney, however increased renal IGF-I levels normalise within 4 days after diabetes induction in the STZ-rat model whereas they remain increased up to four weeks after diabetes onset in the diabetic mouse (5, 12, 15). Renal IGF-I accumulation occurs despite unchanged/reduced renal IGF-I mRNA levels, indicating that IGF-I is taken up from the circulation rather than produced locally (6, 9, 16, 17). IGFBP-1, elevated in diabetic serum, is thought to have a major role in sequestering circulating IGF-I. In the present study, renal IGF-I levels were increased in the diabetic mice and in non-diabetic mice after hIGFBP-1 administration, although renal IGF-I mRNA levels were downregulated or unchanged respectively. Since all samples were collected at day 20 after onset of diabetes, it can not be excluded that a transient increase in renal IGFBP-1 at a time point before day 20, would play a role in the renal IGF-I accumulation seen in the diabetic kidney. Renal IGF-I content was decreased in diabetic mice after hIGFBP-1 administration. Administration of hIGFBP-1 did not change serum IGF-I levels in control animals, but decreased serum IGF-I in diabetic animals. Theoretically, the observed lack of effect of IGFBP-1 administration on renal IGF-I accumulation in diabetic mice could be due to lower serum IGF-I levels combined with very high serum IGFBP-1 levels, leading to reduced renal IGF-I uptake. While renal IGF-I accumulation in diabetic animals coincided with increased kidney weight, increased glomerular volume and impairment of the kidney function reflected in higher UAE, none of these changes were seen in non-diabetic mice receiving hIGFBP-1. Although administration of hIGFBP-1 to diabetic mice did neither affect the increase in kidney weight nor UAE, it abolished the increase in glomerular volume and blunted renal IGF-I accumulation. This suggests that the renal weight and glomerular volume could be affected by different mechanisms. hIGFBP-1 administration may affect other relevant factor operating locally in the glomerulus. In line with this it is of interest that the glomerular hypertrophy, seen in diabetic and high-protein fed animals, is vascular endothelial growth factor (VEGF)-dependent and that VEGF has been suggested to be a downstream mediator of IGF-I effects (18).

Renal expression of IGFBPs was generally unchanged or decreased in the STZ-diabetic mouse. This is in contrast with studies reporting increased protein (30kDa band, IGFBP-1 and/or -2) and mRNA levels of IGFBP-1 in 3-4 weeks diabetic NOD mouse kidney (15, 16, 19). Detailed investigation of IGFBPs mRNA pattern in the diabetic rat kidney, showed a reduction in IGFBP-1 expression in the medulla and increase in the cortex both in short-term (9, 17) and long-term diabetes (9). Our findings are in agreement with studies describing a decrease in renal IGFBP-3 to -5 mRNA levels, both in rat and mouse (9, 15, 17). hIGFBP-1 administration increased IGFBP-1, -2

and –4 protein content, whereas it did not change the IGFBPs mRNA expression pattern in the diabetic kidney. In general unchanged or decreased renal expression of IGFBPs in the STZ-diabetic mouse suggest that IGF-I is captured in the kidney by mechanism not involving IGFBPs.

Immunohistochemical studies detected IGFBP-1 co-localized with IGF-I predominantly in the papillary collecting ducts, but also in cortical collecting ducts and medullary thick ascending limbs of Henle's loop (mTALs) in the rat kidney (8). Immunostainable IGFBP-1 shows the same distribution in the mouse kidney (unpublished data). We were not able to specifically detect exogenous hIGFBP-1 due to lack of a suitable human-specific IGFBP-1 antibody. Although extractable renal IGF-I was decreased in diabetic mice and increased in normal mice following hIGFBP-1 administration, we were unable to clearly see these changes on the immunostained kidney sections. This may be due to the difficulties in using immunohistochemistry for quantification.

Our main aim was to investigate the effect of IGFBP-1 on early changes that take place in the diabetic kidney. Limited availability of purified hIGFBP-1 only allowed us to assess the effect of 2 weeks hIGFBP-1 administration in early diabetes. Although mini-pumps would be more appropriate route of administering hIGFBP-1, they would be insufficient to sustain the required high hIGFBP-1 levels. Since hIGFBP-1 administration in pituitary-deficient Snell dwarf mice resulted in a pronounced increase in kidney weight (152 %) (10), a comparable dose in respect to body weight and the same administration route was used, i.e. 3 daily subcutaneous injections. However, in the present study hIGFBP-1 did not have any major effects, although increased levels of IGFBP-1 were detected in injected mice, both in serum and kidney. It is possible that in Snell dwarf mice, which lack endogenous GH and subsequently have very low IGF-I circulatory levels, administered hIGFBP-1 was able to disturb the fragile balance in the GH/IGF axis, while in normal mice and diabetic mice this was not the case. It may be that changes taking place in diabetic kidney, regarding increased weight and decreased function, are already taking place at a maximal level, so that no effect of IGFBP-1 administration is seen. Further, it cannot be excluded that prolonged administration of hIGFBP-1 would show some additional effect, but in this study we focussed specifically on early renal changes. Another concern might be the phosphorylation state of the hIGFBP-1 preparation used. It has been shown that elevated serum IGFBP-1 in untreated diabetic patients exists in the highly phosphorylated (*p*) state (20), a form that *in vitro* has 4-6-fold higher affinity for IGF-I than the non-phosphorylated (*np*) form (21). Although one might argue that *np*IGFBP-1 is less important than *p*IGFBP-1, a role for *np*IGFBP-1 in the regulation of free IGF-I *in vivo* has been suggested (22). The purified hIGFBP-1 we administered was approximately 50 % phosphorylated and shown to have renal effects in Snell dwarf mice (10). A recent study involving hIGFBP-1 transgenic (TG) mice and rat (r) IGFBP-1 TG mice exhibiting predominantly *np* and *p* form in the serum, respectively, suggested that the phosphorylation state of IGFBP-1 may account for part of the phenotypic differences such as fasting hyperglycaemia and growth restriction in rIGFBP-1 TG but not in hIGFBP-1 TG mice (23). However, another line of hIGFBP-1 TG mice did exhibit growth retardation and glucose intolerance, although to a lesser extent than rIGFBP-1 TG mice suggesting that if predominantly non- or lesser- phosphorylated forms are present in sufficient concentration, hIGFBP-1 could still have an effect (23, 24). It would be interesting in future experiments to study renal changes in diabetic IGFBP-1 TG mice, as well as in IGFBP-1 knock-out (KO) mice.

In conclusion, hIGFBP-1 administration was shown not to have major effects on early renal changes in diabetes. Further studies are needed to describe the alterations in IGFBPs in the diabetic kidney over time and especially to determine their pathophysiological importance. Some of these questions may be addressed in kidney-specific IGFBPs TG and KO diabetic mouse.

ACKNOWLEDGMENTS

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Chapter 4

Dose-response effects of a new growth hormone receptor antagonist (B2036-PEG) on circulating, hepatic and renal expression of the growth hormone/insulin-like growth factor system in adult mice

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Dose–response effects of a new growth hormone receptor antagonist (B2036-PEG) on circulating, hepatic and renal expression of the growth hormone/insulin-like growth factor system in adult mice

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Abstract

The effects of growth hormone (GH) in regulating the expression of the hepatic and renal GH and insulin-like growth factor (IGF) system were studied by administering a novel GH receptor antagonist (GHRA) (B2036-PEG) at different doses (0, 1·25, 2·5, 5 and 10 mg/kg/day) to mice for 7 days. No differences were observed in the groups with respect to body weight, food consumption or blood glucose. However, a dose-dependent decrease was observed in circulating IGF-I levels and in hepatic and renal IGF-I levels at the highest doses. In contrast, in the 5 and 10 mg/kg/day GHRA groups, circulating and hepatic transcriptional IGF binding protein-3 (IGFBP-3) levels were not modified, likely resulting in a significantly decreased IGF-I/IGFBP-3 ratio. Hepatic GH receptor (GHR) and GH binding protein (GHBP) mRNA levels increased significantly in all GHRA dosage groups. Endogenous circulatory GH levels increased significantly in the 2·5 and 5 mg/kg/day GHRA groups. Remarkably, increased circulating IGFBP-4 and hepatic IGFBP-4 mRNA levels were observed in all GHRA administration groups. Renal GHR and GHBP mRNA levels were not modified by GHRA administration at the highest doses. Also, renal IGFBP-3 mRNA levels remained unchanged in most GHRA administration groups, whereas IGFBP-1, -4 and -5 mRNA levels were significantly increased in the 5 and 10 mg/kg/day GHRA administration groups.

In conclusion, the effects of a specific GHR blockade on circulating, hepatic and renal GH/IGF axis reported here, may prove useful in the future clinical use of GHRAs.

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Introduction

The growth hormone (GH)/insulin-like growth factor (IGF) axis constitutes a complex system of peptides in the circulation, extracellular space and in most tissues. The classical endocrine effect of pituitary-secreted GH is the induction of IGF-I synthesis in various organs. The liver is believed to be the major source of circulating IGF-I which, in turn, is a negative feedback signal on GH secretion (Namba *et al.* 1989, Yamasaki *et al.* 1991).

IGF-I is a member of the IGF system that also consists of IGF-II, two types of IGF receptors and six different IGF binding proteins (IGFBPs) (Kelley *et al.* 1996). In addition, four IGFBP-related proteins have been described (Baxter *et al.* 1998).

The GH/IGF axis is important for normal cell growth and differentiation, but has also been shown to be involved in pathophysiological processes. Recently, a series of GH antagonists has been developed that specifically block the GH receptor (GHRAs) (Chen *et al.* 1991*b*, 1994). These GHRAs may be used as intervention in various diseases where GH action is known to play a pathophysiological role (e.g. acromegaly) (Trainer *et al.* 2000) or is suspected of being involved in organ specific damage (e.g. diabetic kidney disease) (Flyvbjerg *et al.* 1999*a*, Segev *et al.* 1999).

In order to get more detailed information about the effects of specific GH receptor (GHR) blockade on circulating and local components of the GH/IGF axis, a GHRA with an enhanced affinity for the human GHR (B2036-PEG) was studied at different doses in mice. The effects of GHRA administration on the expression of the GH/IGF components were investigated in the liver, thought to be the major regulator of circulating IGFs, and in the kidney, as various kidney diseases may be potential targets for GHRA treatment.

Materials and Methods

Animals

Adult female Balb/C(a) mice with initial body weights of 16·4 ± 0·2 g (Bomholtgaard, Ry, Denmark) were used in the study. The animals were housed 7–8 per cage on white special spanwall bedding. They were fed a standard laboratory diet (Altromin No. 1310, Altromin, Lage, Germany), had free access to water and were kept at constant temperature (21 ± 1 °C), humidity (55 ± 5%) and under a 12-h light, 12-h darkness cycle (lights on from 0700 h to 1900 h). The study complied with Danish regulations for the care and use of laboratory animals.

Study design

The mice were randomly allocated into five groups of eight animals: (1) animals injected on days 0, 2, 4 and 6 with 0·154 mol/l NaCl, the vehicle for the other treatments (control group); (2) animals injected on days 0, 2, 4 and 6 with 2·5 mg/kg human GHR antagonist (GHRA): B2036-PEG (1·25 mg/kg/day GHRA group); (3) animals injected on days 0, 2, 4 and 6 with 5 mg/kg GHRA (2·5 mg/kg/day GHRA group); (4) animals injected on days 0, 2, 4 and 6 with 10 mg/kg GHRA (5 mg/kg/day GHRA group); (5) animals injected on days 0, 2, 4 and 6 with 20 mg/kg GHRA (10 mg/kg/day GHRA group).

Human GHRA (B2036-PEG) was kindly provided by Sensus Drug Development Corporation (Austin, TX, USA) (Fuh *et al.* 1992). The molecule has a modification in the first GHR binding site resulting in a 30–50 times increased affinity for the human GHR. In addition, in the second GHR binding site, amino acid 120 is modified preventing GHR dimerization (Chen *et al.* 1991*c*). GHRA was provided in a pegylated formula to warrant prolonged biological action of the molecule (Clark *et al.* 1996). The GHRA was dissolved in 0·154 mol/l NaCl and injected s.c. on days 0, 2, 4, and 6 in an injection volume of 0·5 ml. The animals were weighed and their food consumption and blood glucose was determined on days 0, 2, 4, and 7. On day 7, the animals were anesthetized with pentobarbital (50 mg/kg i.p.) and non fasting blood samples were collected exactly 5 min later from the retrobulbar plexus through heparinized capillary tubes under light ether anesthesia. The serum samples were kept at − 80 °C for later analysis. Whole liver and the left kidney were removed and snap frozen in liquid nitrogen.

Blood glucose

Blood glucose was measured in unanesthetized animals in tail vein blood by Haemoglucotest 1–44 and Reflolux II reflectance meter (Boehringer-Mannheim, Mannheim, Germany).

Serum GH and IGF-I, and hepatic and kidney IGF-I determinations

Serum GH was measured by a radioimmunoassay (RIA) as described previously (Flyvbjerg *et al.* 1999*a*). Potential cross-reactivity of the GHRA (B2036-PEG) with the rodent specific GH assay was precluded, as addition in the assay of the GHRA at multiple concentrations (over a range from 0·5–5000 μg/l) did not reveal any significant binding. Serum IGF-I was measured after extraction using acid-ethanol (Flyvbjerg *et al.* 1999*a*). The intra-assay and interassay coefficients of variation (CV) were 5% and 10% respectively. Tissue extraction of renal and hepatic IGF-I was performed according to D'Ercole *et al.* (1984) and corrected for the contribution of entrapped serum IGF-I (Flyvbjerg *et al.* 1992*b*).

Serum IGFBPs

SDS-PAGE and Western ligand blotting (WLB) analysis were executed according to the method of Hossenlopp *et al.* (1986) as described previously (Flyvbjerg *et al.* 1992*a*).

Gene expression of GH and IGF system (mRNA) in tissues

Gene expression of IGFBP-1 to -6 (mRNA) was measured by Northern blot analysis. Total RNA was extracted from kidney and liver samples by the guanidinium thiocyanate method (Chomczynski & Sacchi 1987). Glyoxylated-RNA samples were electrophoresed in 1% agarose gels submerged in 10 mM sodium phosphate pH 7·2 and transferred to nylon membranes (Hybond N^+, Amersham, 's Hertogenbosch, The Netherlands). Filters were hybridized with 1–2 × 10^6 c.p.m. per ml ^{32}P-labeled cDNA fragments encoding for each of the six mouse IGFBPs (Schuller *et al.* 1994), mouse IGF-I and -II (kindly provided by Dr G I Bell, Howard Hughes Institute, Chicago, IL, USA), rat IGF-I receptor (kindly provided by Dr H Werner and Dr D LeRoith, National Institutes of Health, Bethesda, MD, USA), GHR, GHBP (Mathews *et al.* 1989) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 65 °C according to the method of Church & Gilbert (1984).

Quantification

Autoradiographs of WLBs were scanned using a laser densitometer (Shimadzu model CS 90001 PC, Shimadzu Europe GmbH, Duisburg, Germany) and the relative densities of the bands expressed in pixels. Northern blots were scanned on a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA) and quantified using ImageQuant software. All measured mRNA results were expressed relative to GAPDH mRNA levels.

Statistical analysis

Data were examined for distribution, variance homogeneity (F-test) and analyzed by one-way analysis of variance followed by pair-wise comparisons with the least-significant difference method. All data are expressed as means ± s.e.m., with *n* indicating the number of mice studied. *P* values less than 0·05 are considered significant.

Results

Body weight and organ weights

The body weights of the different study groups at the beginning (day 0) and end (day 7) of the experimental period were determined. The mean body weights on day 0 were 16·4 ± 0·2 g with no differences between the groups. At day 7, mean body weight increased to 18·9 ± 0·3 g in control animals with no differences in the different groups (data not shown). At the end of the experimental period, liver and kidney weights were determined. Liver weight and the weight of the kidneys in the control group were 926 ± 35 mg and 237 ± 3 mg respectively. The GHRA administration regimen demonstrated no significant effects on the weight of liver or kidneys (data not shown).

Food consumption and blood glucose

Food consumption over 24 h was measured during the study period on a group basis. Mean food intake per mouse of the control group was 4·2 ± 0·1 g per 24 h. Food consumption did not differ significantly during the experimental period in any of the groups (data not shown). Blood glucose values were measured in all animals at the end of the experimental period and did not change significantly in any of the experimental groups (data not shown).

Serum IGF-I and GH

At the end of the study period serum IGF-I levels were determined in all groups (Fig. 1A). Compared with placebo control levels, in the 1·25 mg GHRA group serum IGF-I levels were unchanged, whereas in the 2·5, 5 and 10 mg GHRA groups serum IGF-I levels were reduced to 75% (*P*=0·006), 70% (*P*=0·001) and 51% (*P*<0·001) of control values respectively (Fig. 1A). Compared with the other groups significant and dose-dependent decreases in circulatory IGF-I levels were observed (1·25 mg versus 2·5 mg GHRA group, *P*<0·008; 5 mg versus 10 mg GHRA group, *P*=0·011). Figure 1B shows serum GH levels in the five experimental groups by the end of the study (day 7). It has been demonstrated previously that barbital anesthesia induces a marked rise in GH levels that lasts for up to 90 min (Takahashi *et al.* 1971). Therefore, the endogenous GH levels given in Fig. 1B are stimulated values. Increased serum GH levels amounting to 183% (*P*=0·003) and 145% (*P*=0·01) of control values were seen in the 2·5 mg GHRA and 5 mg GHRA groups respectively.

Serum IGFBPs

Using WLB, four distinct bands were obtained. A double band at 38–42 kDa representing IGFBP-3, a single 30 kDa band (IGFBP-1 and -2) and a 24 kDa band identified as IGFBP-4.

GHRA administration did not affect IGFBP-3 levels with the exception of the group receiving 2·5 mg GHRA. In this group IGFBP-3 levels were significantly decreased to 78% of control levels (*P*=0·04) (Fig. 1C). Circulatory 30 kDa (IGFBP-1 and -2) levels were unchanged in all groups (Fig. 1C). IGFBP-4 levels were significantly increased in the 1·25, 2·5 and 5 mg GHRA groups to 143% (*P*=0·008), 142% (*P*=0·04) and 159% (*P*=0·001) of control levels. In the 10 mg GHRA group, IGFBP-4 levels were near significantly increased to 125% (*P*=0·06) of control levels (Fig. 1C). In the 5 and 10 mg GHRA groups the calculated ratio of IGF-I to IGFBP-3 was significantly decreased to 71% (*P*=0·001) and 53% (*P*<0·001) of control values.

Hepatic GH/IGF system expression

GHRA administration for 7 days significantly increased hepatic GHR mRNA expression at all doses tested (Fig. 2B). GHBP mRNA expression was dose-dependently increased in the 1·25 mg, 2·5 mg and 5 mg GHRA groups (control versus 1·25 mg GHRA group, *P*<0·001; 1·25 mg versus 2·5 mg GHRA group, *P*<0·001; 2·5 mg versus 5 mg GHRA group, *P*=0·044). A representative Northern blot is given in Fig. 2A.

Hepatic IGF-I protein levels, however, demonstrated a tendency to decline in all GHRA administration groups, only reaching significance in the 10 mg GHRA group (*P*=0·004) (see Fig. 4A). Hepatic IGF-I mRNA levels (predominantly 1·2 kb and 7 kb) also had a tendency to decline in all GHRA administration groups reaching significance in the 2·5 mg GHRA (*P*=0·01) and 10 mg GHRA (*P*=0·05) groups (Fig. 2C).

Compared with control animals, hepatic IGFBP-1 mRNA levels had a tendency to increase, only reaching significance in the 2·5 mg GHRA group (*P*<0·03) (Fig. 2D). Hepatic IGFBP-2 mRNA levels were significantly decreased in the 1·25, 2·5 and 5 mg GHRA groups (*P*<0·001, *P*=0·002 and *P*=0·001 respectively) (Fig. 2D). IGFBP-3 mRNA levels were unchanged during GHRA administration, whereas IGFBP-4 mRNA levels were significantly increased in all GHRA administration groups (Fig. 2D). Hepatic IGFBP-5 and -6, IGF-II and IGF-I receptor mRNA levels were not detectable in any of the groups.

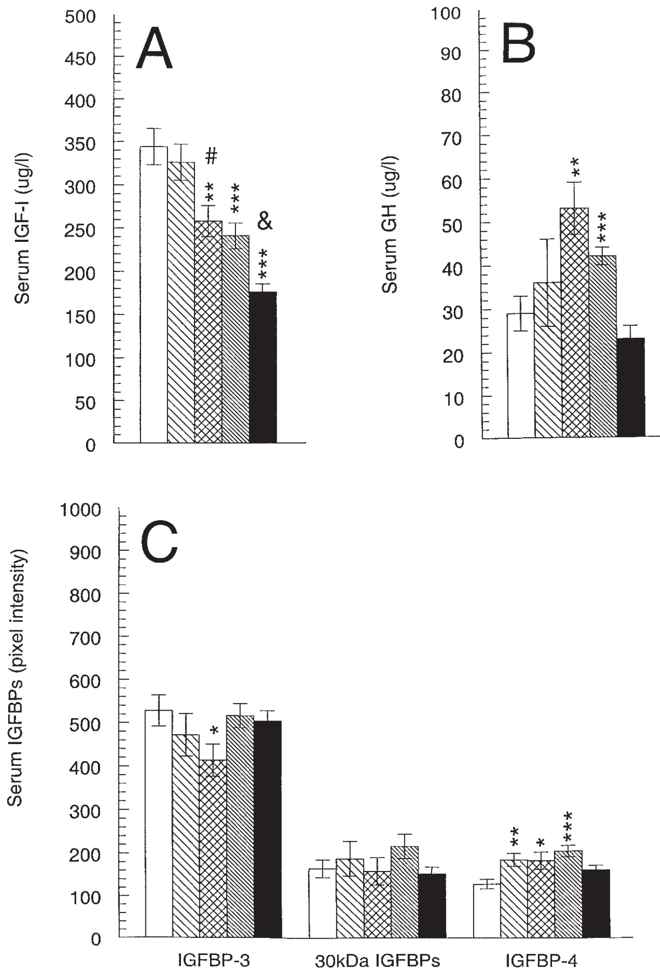
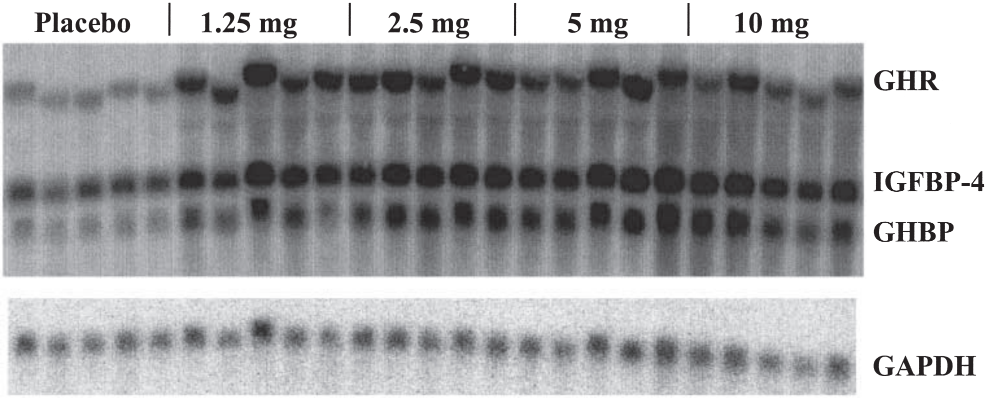


Figure 1 Circulatory levels of (A) IGF-I, (B) GH, and (C) IGFBP in adult mice injected for 7 days with placebo (open bars), 1·25 mg/kg/day (broad-hatched bars), 2·5 mg/kg/day (cross-hatched bars), 5 mg/kg/day (narrow-hatched bars) and 10 mg/kg/day (solid bars) GHRA B2036-PEG. Values are means ± S.E.M. (n=8). *P=0·04, **P<0·01, ***P≤0·001, statistical significance level between the indicated GHRA group and the placebo control. #P=0·008 significance level between the 1·25 mg and the 2·5 mg GHRA group; &P=0·011 significance level between the 5 mg and 10 mg GHRA group.

Renal GH/IGF system expression
GHRA administration did not modify renal GHR mRNA levels except for the 1·25 mg GHRA group where a significantly decreased GHR mRNA level was observed (P=0·03) (Fig. 3B). A representative Northern blot is given in Fig. 3A.

GHRA administration also did not modify renal GHBP mRNA levels in most of the groups. Only the 10 mg GHRA group demonstrated a significantly increased GHBP level (P=0·005) (Fig. 3B).

Compared with the placebo control, in the 5 and 10 mg GHRA groups renal IGF-I protein levels significantly



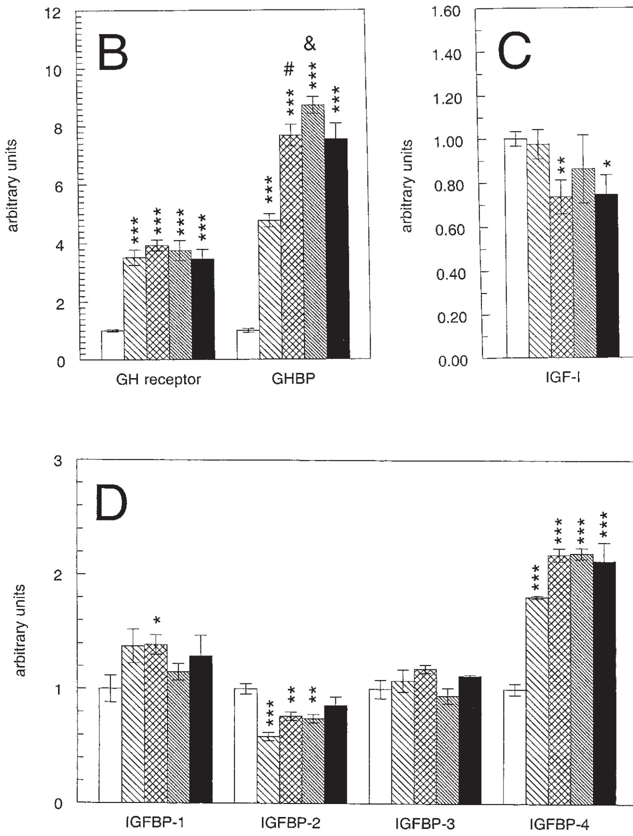
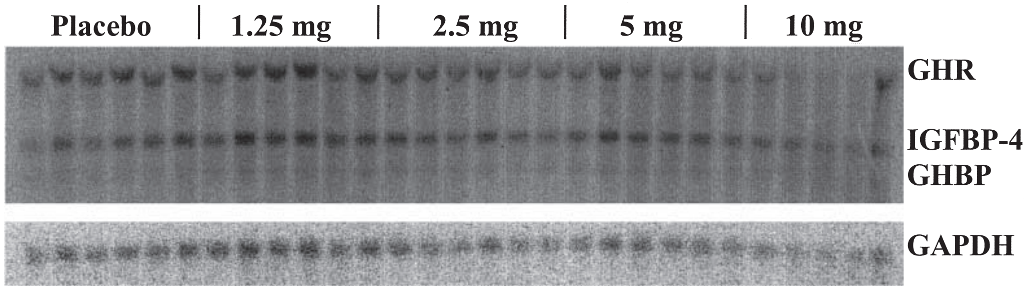
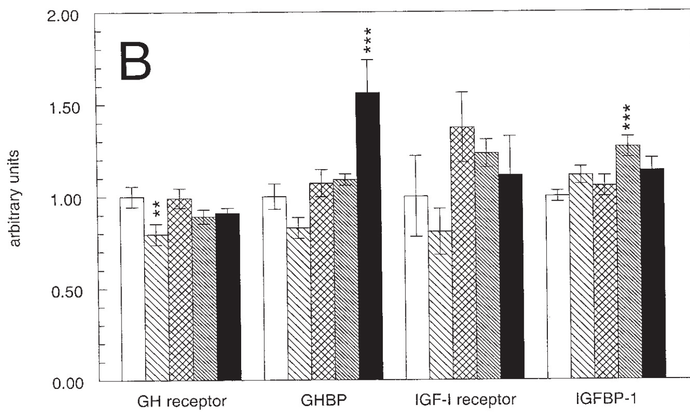


Figure 2 (A) Representative mRNA expression pattern of GHR, GHBP, IGFBP-4 and GAPDH in livers of adult mice injected for 7 days with placebo, 1·25, 2·5, 5 and 10 mg/kg/day GHRA B2036-PEG. (B-D) Relative hybridization of (B) GHR, GHBP mRNA, (C) IGF-I mRNA, and (D) IGFBP-1, -2, -3, -4 mRNA in liver of adult mice injected for 7 days with placebo (open bars), 1·25 mg/kg/day (broad-hatched bars), 2·5 mg/kg/day (cross-hatched bars), 5 mg/kg/day (narrow-hatched bars) and 10 mg/kg/day (solid bars) GHRA B2036-PEG. Values are based on quantitations of Northern blots, compensated for RNA loading differences. Values are represented as means ± S.E.M. (n=8) and expressed relative to the placebo control. *$P \leq 0{\cdot}05$, **$P \leq 0{\cdot}01$, ***$P \leq 0{\cdot}001$, statistical significance level between the indicated GHRA group and the placebo control. $^{\#}P<0{\cdot}001$ significance level between the 1·25 mg and the 2·5 mg GHRA group; $^{\&}P=0{\cdot}044$ significance level between the 2·5 mg and 5 mg GHRA group.





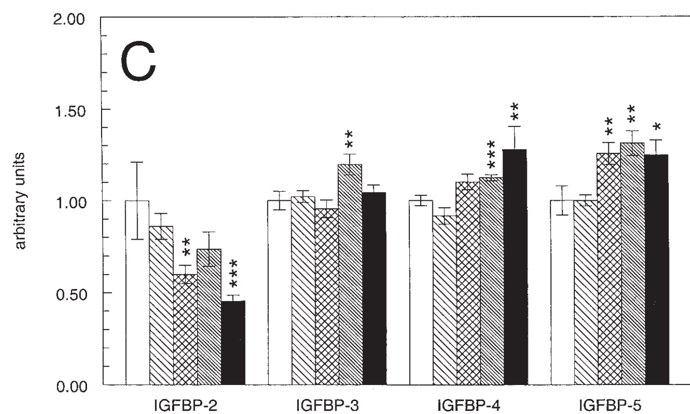


Figure 3 (A) Representative mRNA expression pattern of GHR, GHBP, IGFBP-4 and GAPDH in kidneys of adult mice injected for 7 days with placebo, 1·25, 2·5, 5 and 10 mg/kg/day GHRA B2036-PEG. (B and C) Relative hybridization of (B) GH receptor, GHBP, IGF-I receptor and IGFBP-1 mRNA and (C) IGFBP-2, -3, -4 and -5 mRNA in kidneys of adult mice injected for 7 days with placebo (open bars), 1·25 mg/kg/day (broad-hatched bars), 2·5 mg/kg/day (cross-hatched bars), 5 mg/kg/day (narrow-hatched bars) and 10 mg/kg/day (solid bars) GHRA B2036-PEG. Values are based on quantitations of Northern blots, compensated for RNA loading differences. Values are represented as means ± S.E.M. ($n=8$) and expressed relative to the placebo control. *$P=0·05$, **$P\leq 0·03$, ***$P\leq 0·005$, statistical significance level between the indicated GHRA group and the placebo control.

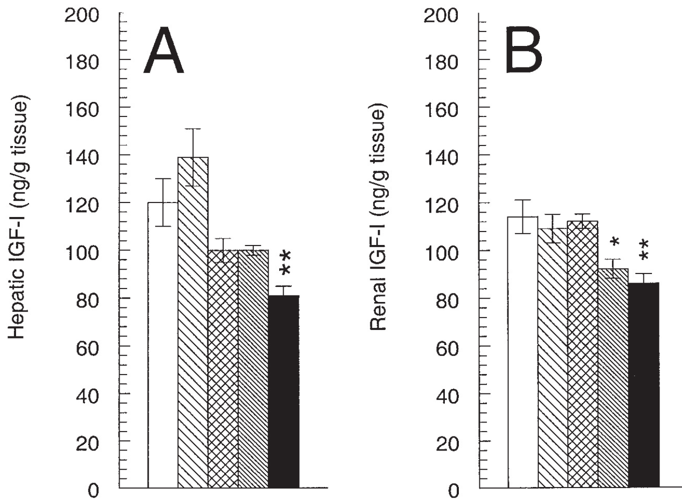


Figure 4 (A) Hepatic and (B) renal IGF-I protein levels in adult mice injected for 7 days with placebo (open bars), 1·25 mg/kg/day (broad-hatched bars), 2·5 mg/kg/day (cross-hatched bars), 5 mg/kg/day (narrow-hatched bars) and 10 mg/kg/day (solid bars) GHRA B2036-PEG. Values are means ± S.E.M. (n=8). *P=0·02, **$P \leq$0·005, statistical significance level between the indicated GHRA group and the placebo control.

decreased (P=0·02 and P=0·005 respectively) (Fig. 4B). IGFBP-1 and -3 mRNA levels were not modified by GHRA administration except for the 5 mg GHRA administration group where a significant increase was observed (P=0·03 and P=0·005 respectively) (Fig. 3C). IGFBP-2 mRNA levels decreased dose-dependently with increasing GHRA concentrations which, however, only reached significance in the 2·5 and 10 mg GHRA groups (P=0·03 and P=0·005 respectively) (Fig. 3C). IGFBP-4 mRNA levels significantly increased in the 5 and 10 mg GHRA groups (P=0·004 and P=0·03 respectively) and IGFBP-5 mRNA levels significantly increased in the 2·5, 5 and 10 mg GHRA groups (P=0·03, P=0·01 and P=0·05 respectively) (Fig. 3C). Renal IGF-I receptor expression did not significantly change with any of the treatments. Renal IGF-I, IGF-II and IGFBP-6 mRNA levels were not detectable in any of the groups.

Discussion

In the present study, for the first time, the specific blockage of GH at the organ level was studied using a novel GHRA (B2036-PEG). B2036-PEG is a member of a GHRA family recently developed for the human GHR that retained its activity in mice. Studies using GHRAs *in vitro* demonstrated high affinity binding to the GH receptor (Chen *et al.* 1991*b*,*c*, Fuh *et al.* 1992). Transgenic animals expressing GHRAs phenotypically resembled dwarf animals with reduced circulating IGF-I levels (Chen *et al.* 1990, 1991*a*). When GHRA transgenic animals were made diabetic, protection to GH- and IGF-I-induced renal damage was observed (Chen *et al.* 1995, 1996). Furthermore, GHRA treatment protected diabetic mice from renal damage (Flyvbjerg *et al.* 1999*a*, Segev *et al.* 1999) and abolished compensatory renal growth in uninephrectomized mice (Flyvbjerg *et al.* 1999*b*).

To elucidate further the effects of GHRA, the present study was performed with exogenous administration of a long-acting formula of GHRA at increasing doses in adult animals. The effects on body weight and on the circulating, hepatic and renal GH and IGF systems were analyzed.

In our study, increasing GHRA doses proportionally decreased circulating and hepatic IGF-I levels. In contrast, circulatory IGFBP-3 levels remained constant. The IGF-I and IGFBP-3 serum values matched hepatic mRNA levels as a dose-dependent decrease in hepatic IGF-I mRNA values was observed, whereas the IGFBP-3 mRNA levels were unchanged at any of the GHRA doses used. In the circulation, this might indicate a decrease in IGF-I bioavailability. In rodents, there is evidence that serum IGFBP-3 is regulated directly by IGF-I and is independent of GH (Clemmons *et al.* 1989, Camacho-Hubner *et al.* 1991). However, in our study circulatory IGFBP-3 protein and hepatic IGFBP-3 mRNA levels remained unchanged despite increased GH and decreased

IGF-I levels. The latter finding is in contrast to the human, where serum IGFBP-3 levels directly reflected GH levels (Blum *et al.* 1993). In addition, patients lacking a functional GHR demonstrated high serum GH and low serum IGF-I and IGFBP-3 levels (Cotterill *et al.* 1992, Gargosky *et al.* 1993). The reasons for these differences in serum IGFBP-3 regulation are unclear.

GHRA administration significantly increased circulatory GH levels, although at the highest GHRA concentration, GH levels were decreased to control values. We cannot readily explain this latter observation. As expected, the increased blocking of the GHRs was reflected by a dose-dependent decrease in circulatory IGF-I levels. Therefore, a dose-dependent increase in circulating GH levels was expected. It could be argued that, due to cross-reactivity of the GHRA and GH, the GHRA interfered in our GH assay. However, this possibility had to be excluded as we could not demonstrate any cross-reactivity between the human GHRA and the mouse GH in the rodent specific RIA used for the measurements of circulatory GH levels. Furthermore, one could argue that a single GH determination is not the best estimate of GH secretion. However, as shown before, a single barbital-stimulated GH measurement may be used as an estimate of GH levels (Takahashi *et al.* 1971).

GHRA administration significantly increased hepatic GHR and GHBP mRNA levels, whereas renal GHR and GHBP mRNA levels did not change significantly. These results are in agreement with other studies examining renal GHR/GHBP mRNA levels (Chen *et al.* 1997, Flyvbjerg *et al.* 1999*b*, Segev *et al.* 1999). Our study may represent a compensatory upregulation of the GHR gene expression in response to the functional blockade of the GHR by the GHRA. However, in contrast to our findings, Chen *et al.* (1997) using GHRA expressing transgenic mice did not observe increased hepatic GHR/GHBP mRNA expression although strongly enhanced liver GHR and serum GHBP protein levels were found in these same GHRA transgenic animals (Chen *et al.* 1997, Sotelo *et al.* 1998).

In GH-deficient dwarf rats, decreased renal IGF-I mRNA and increased renal IGFBP-1 mRNA levels were demonstrated that were normalized with exogenous GH administration (Kobayashi *et al.* 1995). In our study, increased GHRA doses did not have an effect on circulatory 30 kDa IGFBPs or hepatic and renal IGFBP-1 mRNA levels. Remarkably, GHRA administration did increase circulatory IGFBP-4 protein, hepatic IGFBP-4 mRNA and, at higher doses, renal IGFBP-4 mRNA levels also. This stimulation of IGFBP-4 expression, in addition to the decrease in the IGF-I/IGFBP-3 ratio, may lead to a further reduction in IGF-I bioavailability in GHRA-treated animals. Kobayashi *et al.* (1995) also demonstrated increased IGFBP-4 mRNA levels in dwarf rats that, however, were not influenced by GH administration. Therefore, multiple mechanisms may be operative in regulating the expression of this gene.

In summary, in this study we demonstrated that administration of a specific GHRA dose-dependently decreased hepatic and serum IGF-I with no effect on the expression of hepatic or renal IGFBP-1 and -3 levels, while stimulating hepatic and circulatory IGFBP-4 levels, likely creating a significant decrease in IGF-I bioavailability. Our findings provide a framework for understanding the usefulness of GHRA as a therapeutic drug. GHRA treatment may be beneficial in inhibiting the GH/IGF-I axis in diseases in which its involvement is implicated, such as acromegaly and GH/IGF-I sensitive tumors. Furthermore, GHRA administration may influence organ specific, direct, GH action in conditions characterized by renal damage such as diabetic nephropathy.

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Chapter 5

High-protein induced renal enlargement is growth hormone independent

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High-protein induced renal enlargement is growth hormone independent

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High-protein induced renal enlargement is growth hormone independent.

Background. Growth hormone (GH) and insulin-like growth factors (IGFs) have been postulated as pathogenic factors in several forms of renal growth, including that induced by high-protein (HP) diets. Compensatory renal growth (CRG) following renal uninephrectomy is strictly GH dependent, while the exact role of GH as a regulating factor in HP induced renal growth has not been fully clarified.

Methods. To elucidate a possible direct role for GH in HP-induced renal growth, we examined the effect of a newly developed specific GH-receptor (GHR) antagonist (B2036-PEG) on renal growth and renal GH/IGF-system expression in HP-fed mice.

Results. Mice fed a HP diet (45% protein) for one week demonstrated renal hypertrophy and increased renal IGF-I. GH receptor antagonist (GHRA) treatment neither modified renal IGF-I nor abolished the renal hypertrophy. In contrast, however, GHRA administration did modify renal mRNA expression of many members of the GH and IGF systems.

Conclusions. The major new finding is that HP-induced renal growth in adult mice is GH independent.

Insulin-like growth factor (IGF) I has pleiotropic effects on metabolism, cellular proliferation and differentiation that is produced under the influence of growth hormone (GH) [1]. IGF-I is a member of the IGF system that also consists of IGF-II, 2 types of IGF receptors and six different IGF binding proteins (IGFBPs) [2]. In addition, nine IGFBP related proteins have been described [3].

Growth hormone and the IGFs have been implicated as renotropic factors in several forms of renal enlargement such as compensatory renal growth (CRG) following uninephrectomy [4], renal growth following streptozotocin-induced diabetes [5], potassium depletion [6] and high-protein (HP) diets [7].

Insulin-like growth factor-I is a prime candidate for the mediation of renal growth. Rats infused with IGF-I [8] and IGF-I transgenic mice [9] both display glomerular hypertrophy in the setting of high circulating IGF-I levels. In addition, low protein containing (LP) diets have shown to lower circulating IGF-I levels [10] whereas IGF-I was found to be elevated in rats fed a HP diet and displaying renal hypertrophy [7, 11].

The precise involvement of GH in protein-induced hypertrophy is, at present, unclear. In GH-deficient Lewis dwarf rats, renal IGF-I levels and renal growth were unaffected by HP diets [12], suggesting that the renal growth is GH-dependent. In contrast, however, Chin and Bondy demonstrated that HP in GH-deficient rats, displaying a 90% reduction in GH levels, increased renal IGF-I and induced renal hypertrophy, suggesting a GH-independent renal growth [7].

Recently, a series of highly specific GH receptor (GHR) antagonists (GHRAs) has been developed for potential therapeutic use [13–15]. These GHR antagonists have proven to be effective in inhibiting renal enlargement in diabetic mice [14, 16] and in CRG after uninephrectomy [4]. In the present study, we investigated the involvement of GH in HP-induced renal growth using a novel GHRA (B2036-PEG). The effects of HP diet alone and with GHRA administration were investigated with a focus on the renal growth and the circulatory, hepatic and renal expression of the GH and IGF system.

Key words: growth hormone, insulin-like growth factor, high protein diet, kidney, liver, mouse, mRNA.

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METHODS

Animals

Adult female Balb/C(a) mice with initial body weights of 17 to 18 g (Bomholtgaard, Ry, Denmark) were used in the study. The animals were housed 8 per cage on

white special spanwall bedding. Mice had free access to water and were kept at a constant temperature (21 ± 1°C), humidity (55 ± 5%) and a 12-hour light, 12-hour dark cycle (07.00 to 19.00 light). The study complied with Danish regulations for care and use of laboratory animals.

Study design

The mice were randomly allocated into three groups of 16 animals. Eight animals per group were sacrificed at day 2 (d2), and 8 at day 7 (d7). Animals from group 1 [normal-protein (NP) group] were fed a specific synthetic diet containing 18% protein (custom made; Altromin, Lage, Germany). Animals from group 2 [high-protein (HP) group] were fed an isocaloric specific synthetic diet containing 45% protein (custom made, Altromin) and injected with 0.154 mol/L NaCl, the vehicle for the other treatment. Animals from group 3 (HP/GHRA group) were fed an isocaloric-specific synthetic diet containing 45% protein and injected with 2.5 mg/kg human GHRA B2036-PEG at d0, d1, d2, d4, and d6. The rationale for the dose follows that of previous studies [4, 14, 16, 17].

In a second experiment, designed to validate the effectiveness of the GHRA dose used in inhibiting the GH-receptor, 32 mice were randomly allocated to four groups of eight animals. Group 1 duplicated the treatment of the NP group as described earlier; group 2, duplicated the HP group; group 3 were HP 2.5 mg/kg/2 d GHRA injected, as the HP/GHRA group described earlier; group 4 animals were fed an isocaloric-specific synthetic diet containing 45% protein and injected with 20 mg/kg human GHRA B2036-PEG at d0, d1, d2, d4, and d6, and were designated the HP/8×GHRA group.

The GHRA (B2036-PEG) was kindly provided by Sensus Drug Development Corporation (Austin, Texas, USA) [15], dissolved in 0.154 mol/L NaCl and injected SC in a volume of 0.5 mL. The molecule has a modification in the first GHR binding site resulting in a 30 to 50 times increased affinity for the human GHR. In addition, in the second GHR binding site, amino acid 120 is modified preventing GHR dimerization. GHRA was provided in a pegylated formula to warrant prolonged biological action of the molecule [18].

Animals were weighed and their food consumption and blood glucose were determined at days 0, 2 and 7. Before they were sacrificed, the animals were anesthetized with pentobarbital (50 mg/kg IP) and non-fasting blood samples were collected exactly five minutes later from the retrobulbar plexus through heparinized capillary tubes under light ether anesthesia. The serum samples were kept at –80°C for later analysis. Whole liver and the left kidney were removed and snap frozen in liquid nitrogen.

Blood glucose and serum insulin

Blood glucose was measured in unanesthetized animals in tail vein blood by a Haemoglucotest 1-44 and Reflolux II reflectance meter (Boehringer-Mannheim, Mannheim, Germany). Serum insulin was measured by an ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) from DRG Instruments GmbH (Marburg, Germany). Semilog linearity of mouse serum and rat insulin was found at multiple dilutions, indicating antigen similarity between mouse and rat insulin. The intra-assay and inter-assay coefficients of variation (CV) were 5% and 10%, respectively.

Serum IGF-I and kidney IGF-I determinations

Serum IGF-I was measured after extraction using acid-ethanol. The intra-assay and inter-assay CV were 5% and 10%, respectively. Tissue extraction of renal IGF-I was performed according to D'Ercole, Stiles and Underwood [19], and corrected for the contribution of entrapped serum IGF-I [20].

Serum and kidney IGFBPs

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western ligand blotting (WLB) analysis were executed according to the method of Hossenlopp et al [21] as described previously [22].

Gene-expression of IGF system genes (mRNA) in tissues

Northern analysis. Gene-expression was measured by Northern blot analysis. Total RNA was extracted from kidney and liver samples by the guanidinium thiocyanate method [23]. RNA samples were electrophoresed in 1% agarose gels and transferred to nylon membranes (Hybond N^+; Amersham, 's Hertogenbosch, The Netherlands). Filters were hybridized with ^{32}P-labeled cDNA fragments encoding for each of the six mouse IGFBPs [24], mouse acid labile subunit (ALS; kindly provided by Dr. Y.R. Boisclair, Department of Animal Sciences, Cornell University, Ithaca, NY, USA), mouse IGF-I and -II (kindly provided by Dr. G.I. Bell, Howard Hughes Institute, Chicago, IL, USA), rat IGF-I receptor (kindly provided by Dr. H. Werner and Dr. D. LeRoith, National Institutes of Health, Bethesda, MD, USA), GHR/GHBP [25] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 65°C according to the method of Church and Gilbert [26].

Real-time PCR

cDNA synthesis. The RNA preparation was treated with 0.5 units of DNAse I (Invitrogen, Breda, The Netherlands) per μg total RNA for 15 minutes at room temperature. The reaction was terminated by addition of 1 μL of 25 mmol/L ethylenediaminetetraacetic acid (EDTA), followed by heating for 10 minutes at 65°C, after which

it was placed on ice. This mixture was used for reverse transcription. cDNA was synthesized by incubation of 1 μg of total RNA with 1.25 μL of a mixture of oligo$(dT)_{18}$ primers (400 nmol/L)/random hexamers (50 μmol/L) in a total volume of 15 μL for five minutes at 70°C, followed by immediate incubation on ice. To this mixture 5 μL of 5× reverse transcription (RT) buffer, 0.5 μL of 10 mmol · L^{-1} dNTP mix, 1 μL of M-MLV reverse transcriptase (200 U/μL; Promega) and water was added to an end volume of 25 μL. The cDNA synthesis reaction was allowed to proceed for 45 minutes at 37°C, followed by a 15-minute incubation at 42°C. The enzyme was inactivated by heating at 95°C for five minutes.

Determination of mRNA levels. mRNA levels were quantitatively determined on an ABI Prism® 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) using SYBR-green technology. Polymerase chain reaction (PCR) primers for mouse IGF-I (mIGF-I) and mouse hypoxanthine phosphoribosyltransferase (mHPRT) (household gene) were designed using Primer Express 1.5 Software with the manufacturer's default settings. The primers used for mHPRT were: forward primer, TTGCTCGAGATGTCATGAAGGA; reverse primer, AGCAGGTCAGCAAAGAACTTATAG, resulting in an amplicon size of 90 base pairs.

The primer set used for mIGF-I were: forward primer, CCACACTGACATGCCCAAGAC; reverse primer, CCTTCTCCTTTGCAGCTTCGT resulting in an amplicon size of 75 base pairs. In 96-well plates, 12.5 μL SYBR-green master mix was added to 12.5 μL of cDNA (2% of the amount of cDNA derived from a reaction with 1.0 μg of RNA) and 300 nmol/L of forward and reverse primers in water. Plates were heated for two minutes at 50°C and 10 minutes at 95°C. Subsequently, 40 PCR cycles consisting of 15 seconds at 95°C and 60 seconds at 60°C were applied. The absence of genomic DNA contamination in the RNA preparations was confirmed by running RNA samples that had not been subjected to reverse transcription.

Calculation of relative expression levels. Threshold cycles (C_t) were defined as the number of PCR cycles at which the fluorescent signal reached a fixed threshold signal. PCR efficiencies for both primers sets were >95%. Relative expression levels were determined according to the comparative method [27].

Quantification

Autoradiographs of WLBs were scanned using a laser densitometer (Shimadzu model CS 90001 PC; Shimadzu Europe GmbH, Duisburg, Germany) and the relative densities of the bands were expressed in pixels. Northern blots were scanned on a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA) and quantified using ImageQuant software. All measured mRNA results were expressed relative to GAPDH mRNA levels.

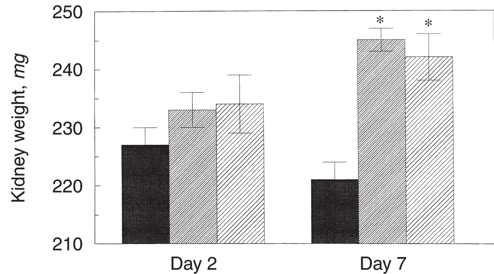

Fig. 1. Kidney weights of mice receiving a normal protein fodder (■), high-protein fodder (▨) and high-protein fodder + GHRA B2036-PEG (▨) for 2 and 7 days. Values are means ± SEM (N = 8). *$P \leq 0.001$, statistical significance level between the indicated group and the respective control.

Statistical analysis

Data were examined for distribution, variance homogeneity (F-test) and analyzed using the Student unpaired *t* test if appropriate. Otherwise, the significance of difference was compared by one-way analysis of variance followed by pair-wise comparisons with the LSD method. All data are expressed as mean ± SEM, with *N* indicating the number of mice studied; *P* values equal to or less than 5% are considered significant.

RESULTS

Body weight, food consumption, blood glucose and serum insulin

The body weights of the different study groups were measured at the beginning (d0) and the end (d2 or d7) of the experimental period. The mean body weight at day 0 was 18.2 ± 0.3 g with no significant differences between the groups. At d2 and d7, the mean body weights of the different groups were not significantly changed by any of the treatments (data not shown). Food consumption over 24 hours was measured during the study period on a group basis. The mean food intake per mouse of the NP group was 3.0 ± 0.1 g/24 h. Food consumption did not differ significantly during the experimental period in any of the groups (data not shown). Also, no changes in blood glucose or insulin levels were seen in any group at any time point (data not shown).

Kidney weight and GH/IGF system expression

At the end of the experimental periods, kidney weights were determined. Kidney weights in the NP group were 227 ± 3 mg and 221 ± 3 mg at d2 and d7, respectively. At d7, the wet-weight of the kidneys was significantly increased in the HP group to 245 ± 2 mg ($P < 0.001$) and in the HP/GHRA group to 242 ± 4 mg ($P < 0.001$; Fig. 1). Related to body weight, both HP and HP/GHRA

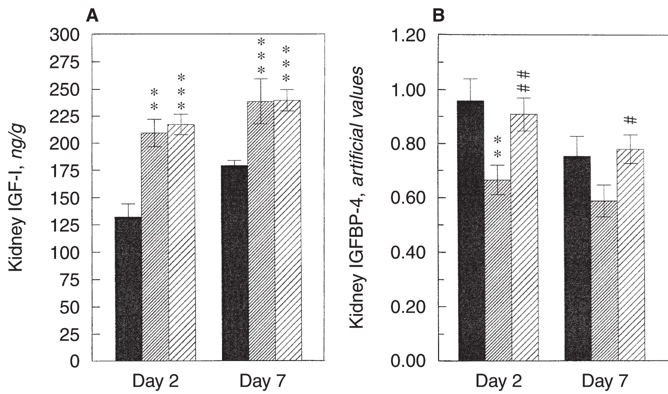

Fig. 2. Kidney IGF-I (μg/L; *A*) and IGFBP-4 (artificial values; *B*) levels of mice receiving a normal protein fodder (■), high-protein fodder (▨) and high-protein fodder + GHRA B2036-PEG (▨) for 2 and 7 days. Values are means ± SEM (N = 8). $^{**}P \leq 0.01$, $^{***}P \leq 0.002$, statistical significance level between the indicated group and the respective control. $\#P = 0.04$, $\#\#P = 0.01$, statistical significance level between the high-protein/GHRA group and the high-protein group.

administration significantly increased the weight of the kidneys at d2 and d7: P = 0.005 and P < 0.001 at d2 and P < 0.001 and P < 0.001 at d7, for HP and HP/GHRA, respectively.

Compared to the NP group, renal IGF-I levels were significantly increased in the HP groups at d2 (P = 0.01) and d7 (P = 0.002). GHRA administration did not further modify renal IGF-I levels (Fig. 2A).

Using WLB, four distinct IGFBP bands were obtained. A double band at 38-42 kD representing IGFBP-3, a single 30 kD band (IGFBP-1 and -2) and a 24 kD band identified as IGFBP-4. Renal 30 kD IGFBPs (IGFBP-1 and -2) and IGFBP-3 levels were unchanged in all groups (results not shown), whereas renal IGFBP-4 levels were significantly decreased in the HP group at d2 (P = 0.01) but not at d7 (Fig. 2B). Compared to the HP group, GHRA administration increased IGFBP-4 levels both at d2 and d7 (P = 0.01 and P = 0.04, respectively; Fig. 2B).

Renal GHBP mRNA levels were significantly reduced in the HP group at d2 (P = 0.005) when compared to the NP group. At d7 both GHR and GHBP mRNA levels were significantly reduced in the HP group (P < 0.003 and P = 0.004, respectively). GHRA administration for 7 days significantly increased GHR and GHBP mRNA levels (P = 0.01 and P = 0.03, respectively; Fig. 3A).

Renal IGF-I mRNA levels were not significantly altered in any of the groups (results not shown). IGFBP-1 mRNA levels decreased in the HP/GHRA group both at d2 and d7 (P = 0.007 and P < 0.001, respectively), whereas the levels in the HP group remained unchanged (results not shown). In the HP and HP/GHRA groups IGFBP-2 mRNA levels were significantly decreased after 7 days (P = 0.02) without an additional effect of GHRA administration (results not shown). HP feeding did not change renal IGFBP-3 mRNA levels. In contrast, GHRA administration significantly reduced IGFBP-3 mRNA levels both at d2 and d7 (P = 0.008 and P < 0.03, respectively, and when compared to the HP group; Fig. 3B). In the HP group, IGFBP-4 mRNA levels significantly decreased at d2 and d7 (P < 0.001 and P < 0.04, respectively). At d2, GHRA administration did not have an additional effect. However, at d7, GHRA significantly increased hepatic IGFBP-4 mRNA levels (P = 0.001; Fig. 3B). IGFBP-5 mRNA levels were not modified by HP and/or GHRA administration (results not shown).

Renal IGFBP-6, IGF-I, IGF-II and IGF-I receptor mRNA levels were not detectable in any of the groups.

Circulating IGF-I and IGFBPs, liver weight and liver GH/IGF system mRNA expression

At the end of the study periods, circulatory IGF-I levels were determined in all groups. In the NP group, circulatory IGF-I levels were 236 ± 24 μL/L at d2 and 226 ± 12 μL/L at d7 and remained unchanged in the HP and HP/GHRA groups (results not shown). IGFBP-3 levels were increased in the HP group at d2 (P = 0.02) but remained unchanged at d7. Circulatory 30 kD IGFBPs (IGFBP-1 and -2) and IGFBP-4 levels were unchanged in all groups (results not shown).

Liver weight in the NP group was 968 ± 78 mg. Liver weights were not significantly modified in any of the treatment groups (data not shown).

Compared to the NP group, hepatic GHR and GHBP mRNA levels were significantly reduced in the HP group both at d2 (P < 0.001) and d7 (P < 0.001). Also in the HP/GHRA group, GHR and GHBP mRNA levels were significantly reduced at d2 (both P < 0.001). However, GHRA administration for 7 days resulted in significantly increased GHR and GHBP mRNA levels (P < 0.001) (Fig. 4A).

Hepatic IGF-I mRNA levels were significantly reduced in the HP group both at d2 and d7 (P < 0.001

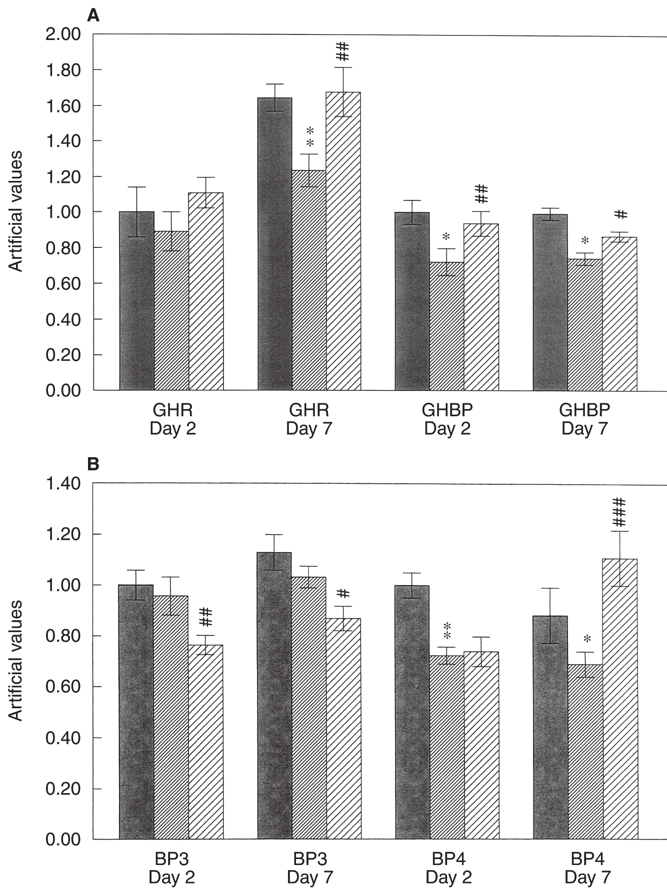

Fig. 3. (*A*) Renal GH-receptor (GHR) and GH-binding protein (GHBP) mRNA levels in adult mice fed for 2 and 7 days with normal-protein fodder (■), high-protein fodder (▨), and high-protein fodder + GHRA B2036-PEG (▨). Values are based on quantitations of Northern blots, compensated for RNA loading differences. Values are means ± SEM ($N = 8$). $*P < 0.007$, $**P < 0.003$; statistical significance level between the high-protein group and the normal-protein group. $\#P = 0.03$, $\#\#P \leq 0.01$; statistical significance level between the high-protein group and the high-protein/GHRA group. (*B*) Renal IGFBP-3 and -4 mRNA levels in adult mice fed for 2 and 7 days with normal-protein fodder (■), high-protein fodder (▨), and high-protein fodder + GHRA B2036-PEG (▨). Values are based on quantitations of Northern blots, compensated for RNA loading differences. Values are means ± SEM ($N = 8$). $*P = 0.04$, $**P < 0.001$; statistical significance level between the high-protein group and the normal-protein group. $\#P < 0.03$, $\#\#P = 0.008$, $\#\#P = 0.001$; statistical significance level between the high-protein group and the high-protein/GHRA group.

and $P = 0.008$, respectively). However, GHRA administration for 7 days significantly increased the IGF-I mRNA level to NP values ($P = 0.02$; Fig. 4B).

Acid labile subunit (ALS) mRNA levels also were significantly reduced in the HP group both at d2 and d7 ($P < 0.001$) and increased to NP levels in the HP/GHRA group at d7 (Fig. 4B)

Compared to the NP group, hepatic IGFBP-1 and -2 mRNA levels had a tendency to decrease after 2 days of HP feeding, however, significance for IGFBP-2 was only reached in the HP/GHRA group ($P = 0.001$). In contrast, at d7, IGFBP-1 and -2 mRNA levels were comparable in all groups (results not shown). IGFBP-3 mRNA levels were significantly reduced in the HP group at d2 ($P = 0.001$), without an additional effect of GHRA administration. In contrast, at d7, IGFBP-3 mRNA levels were increased to control values in the HP/GHRA group ($P = 0.001$; Fig. 4B). IGFBP-4 mRNA levels significantly increased at d2 in both the HP and the HP/GHRA group ($P = 0.01$ and $P = 0.03$, respectively). However, at d7, strongly increased hepatic IGFBP-4 mRNA levels (to 2.05 ± 0.04; $P < 0.001$) only could be observed in the HP/GHRA group (results not shown).

Hepatic IGFBP-5 and -6, IGF-II and IGF-I receptor mRNA levels were not detectable in any of the groups.

To further validate the GH-independence of the HP-induced renal enlargement, we repeated the experiment and included a fourth group consisting of an eightfold increased dose of the antagonist (HP/8×GHRA group; Table 1). Circulating IGF-I and hepatic IGF-I mRNA were significantly lowered in this HP/8×GHRA group (both $P < 0.001$). For body weight, kidney weight, renal IGF-I and IGF-I mRNA and IGFBP-3, no significant

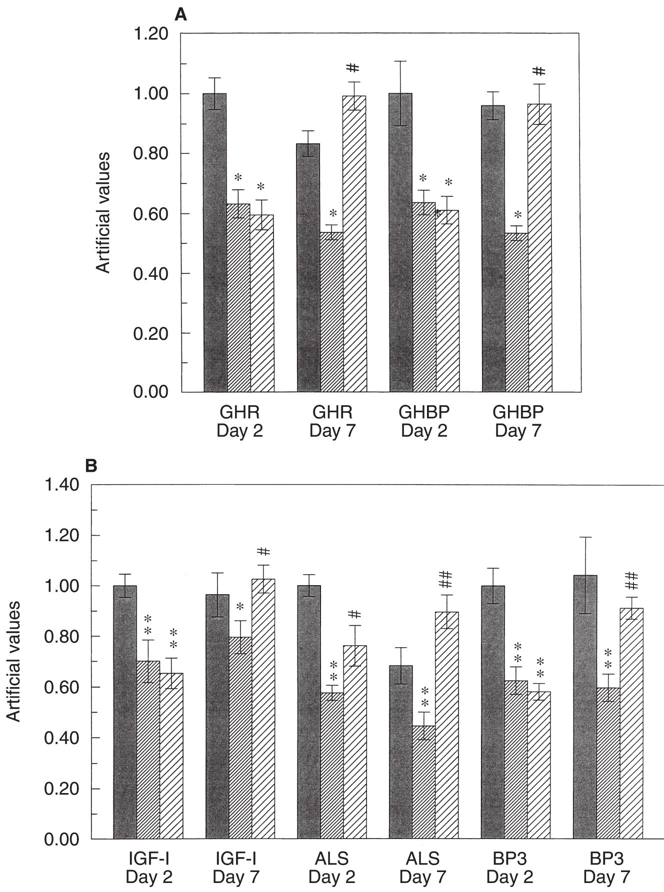

Fig. 4. (*A*) Liver growth hormone receptor (GHR) and GH-binding protein (GHBP) mRNA levels in adult mice fed for 2 and 7 days with normal-protein fodder (■), high-protein fodder (▨), and high-protein fodder + GHRA B2036-PEG (▨). Values are based on quantitations of Northern blots, compensated for RNA loading differences. Values are as means ± SEM ($N = 8$). *$P \leq 0.001$, statistical significance level between the high-protein group and the normal-protein group. #$P \leq 0.001$, statistical significance level between the high-protein/GHRA group and the high-protein group. (*B*) Liver IGF-I, acid labile subunit (ALS) and IGFBP-3 mRNA levels in adult mice fed for 2 and 7 days with normal-protein fodder (■), high-protein fodder (▨), and high-protein fodder + GHRA B2036-PEG (▨). Values are based on quantitations of Northern blots, compensated for RNA loading differences. Values are means ± SEM ($N = 8$). *$P = 0.008$, **$P \leq 0.001$, statistical significance level between the high-protein group and the normal-protein group. #$P < 0.02$, ##$P < 0.001$, statistical significance level between the high-protein/GHRA group and the high-protein group.

Table 1. Repeated experiment with a fourth group

	Normal protein	High protein	HP/GHRA	HP/8 × GHRA
Body weight *g*	19.1 ± 0.5	18.7 ± 0.3	18.5 ± 0.4	18.4 ± 0.3
Kidney weight *mg*	217 ± 4	241 ± 2[c]	246 ± 3[c]	247 ± 5[c]
Kidney IGF-I *ng/g*	168 ± 7	203 ± 8[b]	197 ± 5[b]	195 ± 2[b]
Kidney IGF-I mRNA *arbitrary units*	100 ± 10	85 ± 8	86 ± 8	80 ± 14
Circulating IGF-I	248 ± 18	252 ± 13	236 ± 7	178 ± 5[b,d,e]
Liver IGF-I mRNA *arbitrary units*	100 ± 8	153 ± 25	143 ± 10[b]	79 ± 6[a,e]
Circulating IGFBP-3 *arbitrary units*	1378 ± 41	1300 ± 90	1514 ± 154	1345 ± 87

Body weight, kidneys weight, kidney insulin-like growth factor-I (IGF-I), kidney IGF-I mRNA, circulating IGF-I, liver IGF-I mRNA and circulating insulin-like growth factor binding protein (IGFBP-3) levels in adult mice fed for 7 days with normal-protein fodder, high-protein fodder, high-protein fodder + 1.25 mg/kg/day growth hormone receptor antagonist B2036-PEG (HP/GHRA), and high-protein fodder + 10 mg/kg/day GHRA B2036-PEG (HP/8×GHRA) mRNA values are based on real-time quantitative PCRs, compensated for differences in starting amount. Values are means ± SEM ($N = 8$).

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, statistical significance level between the indicated group and the normal-protein group

[d] $P \leq 0.001$, statistical significance level between the high-protein group and the HP/GHRA group

[e] $P \leq 0.001$; statistical significance level between the HP/GHRA group and the HP/8 × GHRA group

change could be observed between the HP/GHRA and HP/8×GHRA group (Table 1).

DISCUSSION

The major new finding of this study is that the well-known HP-induced renal enlargement is not GH-dependent.

The relationship between GH–IGF-I, food intake and renal growth has attracted much attention. Short-term fasting and long-term starvation both reduced circulatory IGF-I levels [28, 29] and renal mass [30], and accordingly it was suggested that IGF-I (circulating and/or renal) contributed to the renal mass increase. Also, in fasted GH-deficient rats, renal IGF-I levels were reduced whereas renal IGFBP-1 levels were increased [31]. Administration of high-dose GH to these animals restored hepatic IGF-I mRNA abundance, however, did not produce a normal increase of the circulatory IGF-I concentration [32]. In humans, protein-energy malnutrition is accompanied by a high circulatory concentration of GH that also fails to maintain IGF-I in the normal range [32]. Increased dietary protein feeding demonstrated the opposite effect, that is, increased renal IGF-I levels with HP-induced renal growth [11]. In rats with partial nephrectomy, HP-diets even further increased renal IGF-I [17, 33, 34]. However, dietary protein content did not influence renal hypertrophy and renal IGF-I levels in GH-deficient dwarf rats [12, 34]. This suggested a GH-dependence of the HP-induced renal growth. In contrast, however, other studies using GH-deficient animals have described HP-induced renal growth and renal IGF-I, suggesting GH-independence [7, 35].

Our study directly blocked the effects of GH at the receptor level, and demonstrated a HP-induced renal growth independent of the GH-status. The GHRA was used at a concentration that did not significantly modify circulatory IGF-I [4, 14, 16, 36]. However, we had all signs of a GHR blockade at the hepatic and renal level, such as increased hepatic and renal IGFBP-4 mRNA and protein levels, and GHR/GHBP mRNA levels [36]. In the rat, alternative splicing of a single transcript results in a distinct GHR and GHBP mRNAs [37]. The observed increased GHR mRNA levels likely represented a compensatory up-regulation of the GHR gene expression in response to the functional blockade of the GHR by the GHRA. Furthermore, GHRA at this concentration has been proven to block GH-effects on kidneys [4, 14, 16]. This was further verified in a second experiment where a high dose of the GHRA was used that significantly lowered circulatory IGF-I. Also, at this dose a HP-induced renal growth could be demonstrated indicating its GH-independence.

Renal IGFBP-1 and IGFBP-3 mRNAs were reduced when GHRA was added to the HP group. In contrast, renal IGF-I increased to supranormal levels irrespective of the presence of the GHRA. In the liver, HP feeding decreased ALS and IGFBP-3 mRNA levels. In contrast, GHRA addition increased ALS and IGFBP-3 mRNA levels. Low protein feeding for 7 days did not affect ALS or IGFBP-3 mRNA levels [38]. In the kidney, the observed increased IGF-I levels likely created an increase in IGF-I bioavailability that may have contributed to the observed increase in renal weight. In the liver, unchanged IGF-I and increased IGFBP mRNA levels caused a decrease in IGF-I bioavailability that did not modify liver weight.

Growth hormone and IGF-I also have been implicated in other pathophysiological conditions with increased renal growth, such as diabetes and CRG following uninephrectomy. Here, GHRA administration at a concentration also used in this study, inhibited the renal weight increase and glomerular hypertrophy and reduced renal IGF-I [4, 14]. This suggests that in these pathological conditions renal GH-action is a prerequisite for the renal effects. In contrast, GHRA administration did not abolish HP-induced renal enlargement as shown in this study.

The HP model may be important for understanding the role of IGF-I in physiological processes, and GHRA additionally may provide insight in the effects of blocking locally produced renal IGF-I. In our study, renal IGF-I was significantly elevated, irrespective of a GHR blockade. We can only speculate about the mechanism that leads to the increased renal IGF-I levels. It may be that HP directly increased renal transcription of the IGF-I gene, although on our Northern blots, IGF-I mRNA levels were not detectable in any of the groups. However, Chin and Bondy, using in situ hybridization in GH-deficient rats, demonstrated increased IGF-I mRNA levels in the medullary thick ascending limb in response to HP feeding [7]. Alternatively, post-transcriptional regulation may underlay the increased IGF-I levels. In this context, Straus and Takemoto studied dietary protein restriction and demonstrated a greater decrease in the hepatic 7.5 kb IGF-I mRNA than the other IGF-I mRNAs [39]. Finally, it also may be that the changed IGFBP profiles attracted IGF-I to the kidney. Especially the role of IGFBP-1 deserves further attention, as it is implicated in mechanisms resulting in increased renal weight. In a recent study by Doublier et al, using IGFBP-1 transgenic mice, renal glomerular hypertrophy was observed although with unaltered renal weight [40]. In another recent study by van Buul-Offers et al, IGFBP-1 was administered to Snell dwarf mice and an increased renal weight was observed, whereas the weights of most other organs and body weights were inhibited [41]. In our study, hepatic IGFBP-1 mRNA levels did not change in any of the groups. However, the unmodified 30 kD band in the WLB consists of multiple IGFBPs (IGFBP-1, -2 and/or -5). Therefore, we cannot exclude increased circulatory

IGFBP-1 levels, as antisera detecting rodent IGFBP-1 using Western analysis to our knowledge are not available. However, glucose and insulin levels, known factors involved in the regulation of IGFBP-1 were also unchanged. Although we cannot exclude a renal weight increase caused by a non-GH-IGF-I dependent mechanism, it is striking that both groups displaying increased renal IGF-I levels also demonstrate an increased renal weight.

In summary, we demonstrate that specifically blocking the GHR has no impact on HP-induced renal growth, indicating the process to be GH independent.

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Chapter 6

Administration of human insulin-like growth factor binding protein-1 increases circulating levels of growth hormone in mice

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Endocrinology 145:4401–4407, 2004

Administration of Human Insulin-Like Growth Factor-Binding Protein-1 Increases Circulating Levels of Growth Hormone in Mice

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GH is the major regulator of circulating IGF-I, which, in return, controls pituitary GH secretion by negative feedback. IGF-binding protein-1 (IGFBP-1) is believed to modify this feedback through its effects on free IGF-I. In the present study we investigated the potential influence of IGFBP-1 on GH secretion in the absence or presence of a GH receptor antagonist (GHRA) that specifically blocks peripheral GH action. We administered human (h) IGFBP-1 and GHRA to mice alone or in combination for 2 or 7 d. GHRA was administered in a dose previously shown to block GH action without an effect on circulating GH or IGF-I levels. hIGFBP-1 administration increased stimulated circulating GH levels and serum total IGF-I and IGFBP-3 levels. Coadministration of GHRA abolished the hIGFBP-1-induced increase in serum IGF-I and IGFBP-3 levels, whereas stimulated GH levels remained increased. Free IGF-I levels in serum were unchanged in all treatment groups. In conclusion, GH serum levels increased in response to hIGFBP-1 administration, even in the setting of normal IGF-I levels. This finding suggests a direct involvement of IGFBP-1 in GH secretion. (*Endocrinology* 145: 4401–4407, 2004)

GH, RELEASED FROM anterior pituitary somatotrophs, is a major participant in growth and metabolism. The primary regulators of GH synthesis and secretion are two hypothalamic peptides: GHRH and somatostatin (SS). GH itself can regulate its own production at the hypothalamic level, where it modulates the release of GHRH and SS (1), and at pituitary level, where it has an autocrine inhibitory effect on secretion from the somatotroph (2). GH secretion is also regulated through a negative feedback, through circulating IGF-I (3, 4).

The IGF family consists of a complex system of peptides involving two growth factors (IGF-I and IGF-II), two types of IGF receptors, and six high affinity IGF-binding proteins (IGFBPs), which serve as important regulators of IGF bioactivity (5). Under normal conditions, circulating GH is bound to a high affinity GH-binding protein (GHBP), which represents the extracellular domain of the GH receptor (GHR) (6). Most of the circulating IGF-I is bound as a 150-kDa ternary complex that includes IGF-I, IGFBP-3, and acid-labile subunit (ALS). The remaining fraction of IGF-I circulates either as free peptide or bound in a binary complex with one of five other IGFBPs. In addition to stimulating IGF-I synthesis in the liver, GH promotes the formation of IGFBP-3, the most abundant circulating IGFBP, and ALS (7). GH suppresses transcription of IGFBP-1 *in vitro* (8). Serum IGFBP-1 concentrations show an inverse relationship to GH status (9–13), but it is generally believed that such an effect is secondary to a GH-induced increase in insulin levels (14). However, a suppressive effect of GH on IGFBP-1 appears to be unmasked in the presence of low insulin levels (15).

Whether IGFBP-1 has a direct feedback effect on GH is unknown. The GH-deficient Snell dwarf mouse is not an ideal model to address questions about the effect of IGFBP-1 on the GH/IGF axis because it exhibits combined pituitary hormone deficiencies (16). Accordingly, we decided to examine the effect of exogenously administered human (h) IGFBP-1 to intact mice. To specifically block peripheral GH action at the receptor level, we used a GHRA [B2036-polyethylene glycol (PEG)], an analog of GH with altered binding properties preventing GHR dimerization and PEG-modified to prolong its action (17, 18). This GHRA blocks peripheral GH action without enhancing GH secretion (19). Recently, administration of this GHRA in mice was shown to reduce serum IGF-I levels in a dose-dependent manner (18). In the present study, we used a GHRA dose shown not to alter serum IGF-I, IGFBP-3, or GH levels or renal and hepatic IGF-I contents (18), but still with a GHR-blocking effect (20, 21).

The main aim of the present study was to determine whether hIGFBP-1 has direct feedback on GH secretion. In addition, we studied the effect of hIGFBP-1 administration

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Abbreviations: ALS, Acid-labile subunit; GHBP, GH-binding protein; GHR, GH receptor; GHRA, GH receptor antagonist; GHRH, GH-releasing hormone; h, human; IGFBP-1, IGF-binding protein-1; LID, liver IGF-I-deficient; m, mouse; PEG, polyethylene glycol; SS, somatostatin; TG, transgenic; WLB, Western ligand blot.

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on gene expression of IGFBP-1 and IGF-I and their protein levels in liver and kidney.

Materials and Methods

hIGFBP-1 isolation and purification

hIGFBP-1 was isolated from midgestational amniotic fluid obtained for diagnostic purposes with approval of the ethical committee (Sophia Children's Hospital, Rotterdam, The Netherlands). Filtered amniotic fluid was precipitated overnight with 1.8 M ammonium sulfate. After centrifugation, the pellet was dissolved in 50 mM Tris-HCl (pH 7.5). This was centrifuged again, and MeOH was added to the supernatant to a final concentration of 65% and incubated for 30 min at room temperature before a second centrifugation. The supernatant was separated on a C18 Sep-Pak column (Waters Chromatography Division, Millipore Corp., Bedford, MA), and 0.6 vol acetone was added for an overnight precipitation at −20 C. After centrifugation, the air-dried pellet was dissolved in 30% MeOH/50 mM Tris, pH 8.2 (Fig. 1A, lane 2), and further purified on a RESOURCE RPC 3-ml column connected to a fast protein chromatography system (Automatic FPLC; Pharmacia Biotech, Uppsala, Sweden) using a 48–90% methanol gradient (22). hIGFBP-1 fractions were pooled and examined for purity on SDS-PAGE (Coomassie Blue stain), followed by Amido Black staining and immunostaining of Western blots (Fig. 1A, lane 1) (23). Acetone (0.6 vol)-precipitated pellets were dissolved in 10 mM NH_4HCO_3, freeze-dried (Edwards Modulyo, Crawley, UK) and stored at −20 C. The binding of purified hIGFBP-1 was tested by incubating aliquots of hIGFBP-1/0.154 M NaCl at 37 C for up to 9 h, which were subsequently visualized on Western ligand blots (WLB) with ^{125}I-labeled IGF-II (24, 25) (Fig. 1B).

Animals and study design

Adult female BALB/c (a) mice (Taconic M&B, Ry, Denmark), 8 wk old, with an initial body weight of 17–18 g were used. The mice were housed seven per cage in a room with a 12-h light, 12-h dark cycle at constant temperature (22 ± 2 C) and humidity (55 ± 2%). They had free access to tap water and standard food pellets (Altromin 1324, Lage, Germany). Animal handling complied with Danish regulations for laboratory animals.

The study consisted of three parts: experiment 1 (7-d duration), experiment 2, and experiment 3 (2-d duration each). In each experiment the animals were randomized into four groups and injected with equal volumes of 1) placebo (0.154 M NaCl; n = 7); 2) GHRA (n = 7); 3) hIGFBP-1 (n = 7); and 4) hIGFBP-1/GHRA (n = 7). hIGFBP-1 was administered by three daily sc injections (0800, 1600 and 2400 h) in a dose of 120 μg hIGFBP-1/mouse/d for 7 d (experiment 1) or 2 d (experiments 2 and 3). Placebo groups received an equivalent volume of vehicle (0.154 M NaCl). GHRA (B2036-PEG; Pfizer, New York, NY) was dissolved in 0.154 M NaCl, and 2.5 mg/kg were injected sc on d 0, 2, 4, and 6 in experiment 1 or on d 0 and 1 in experiments 2 and 3 [1.25 mg GHRA/kg/d) (18)]. The animals were weighed, and their food consumption and blood glucose levels (Precision Xtra; Abbott Laboratories, MediSense Products, Bedford, MA) were determined. In experiment 1, serum GH was measured on d 7. Because no significant differences between the groups were seen, in experiment 2 we measured circulatory GH levels before and 10 min after stimulation by the GH secretagogue, ipamorelin (Novo-Nordisk A/S, Bagsvaerd, Denmark). Ipamorelin was administered ip in a dose of 40 μg/600 μl/mouse (26). Experiment 3 was performed following the same protocol as that used in experiment 2, except without ipamorelin stimulation to avoid interference with GH/IGF system mRNA measurements.

Animals were killed on d 7 (experiment 1) or d 2 (experiments 2 and 3), exactly 2.5 h after the last hIGFBP-1 injection. Blood samples were collected from the retroorbital venous plexus through heparinized capillary tubes, exactly 5 min after sodium barbital (10 mg/kg body weight, ip) anesthesia. The serum samples were kept at −80 C until analysis. Immediately after bleeding, kidneys and liver were weighed, snap-frozen in liquid nitrogen, and stored at −80 C.

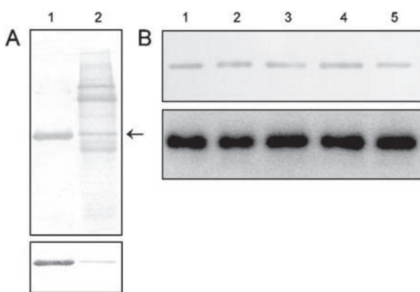

FIG. 1. hIGFBP-1 purification (A). Material loaded onto a RESOURCE RPC/ FPLC column (lane 2) and purified hIGFBP-1 (lane 1) was examined by Amido Black staining (*upper panel*) and immunostaining (*lower panel*) of Western blots. The *arrow* indicates the hIGFBP-1 band. The binding of purified hIGFBP-1 (B) incubated for 0, 2, 4, 6, and 8 h (lanes 1–5, respectively) at 37 C was examined by immunostaining of Western blots (*upper panel*) and by WLB (*lower panel*).

Immunoassays

Serum GH, serum total IGF-I, and tissue IGF-I (liver and kidney) were measured by RIA as described previously (20). For measurement of free IGF-I (27), serum was pooled from two or three animals to achieve a volume sufficiently large to allow determination by ultrafiltration. The pooled serum samples were ultrafiltered without preceding dilution. Serum insulin was measured by an ultrasensitive rat insulin enzyme-linked immunosorbent assay (DRG Diagnostics, Marburg, Germany). Semilog linearity of mouse serum and rat insulin was found at multiple dilutions, indicating antigen similarity between mouse and rat insulins. The intra- and interassay coefficients of variation were less than 5% and less than 10%, respectively, for the GH, total IGF-I, and insulin assays. The within- and between-assay coefficients of variation of free IGF-I were both 20%. Complete removal of serum and tissue IGFBPs was verified by WLB (see below) and Western immunoblotting with a polyclonal goat IGFBP-1 antibody or a polyclonal goat IGFBP-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in a dilution of 1:1000. The IGFBP-1 antibody cross-reacts with both human and mouse (m) IGFBP-1.

WLB

Serum, liver, and kidney IGFBPs were analyzed by SDS-PAGE and WLB as described previously (28). Autoradiographs of WLBs were quantified using a Shimadzu CS-9001 PC dual wavelength, flying spot scanner (Shimadzu Europe GmbH, Duisburg, Germany). The relative densities of the bands were expressed in pixels.

Real-time PCR

Total RNA was extracted from liver and kidney samples by TRIzol reagent (Invitrogen Life Technologies, Gaithersburg, MD) (29). Hepatic and renal GH/IGF system mRNA levels were determined by quantitative real-time PCR (TaqMan) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR-Green technology, as described previously (30). The primers used for mouse hypoxanthine phosphoribosyltransferase (household gene) were: forward primer, TTGCTCGAGATGTCATGAAGGA; and reverse primer, AGCAGGTCAGCAAAGAACTTATAG, resulting in an amplicon size of 90 bp. The primer set used for mIGF-I was: forward primer, CCACACTGACATGCCCAAGAC; and reverse primer, CCTTCTCCTTTGCAGCTTCGT, resulting in an amplicon size of 75 bp. The primer set used for mIGFBP-1 was: forward primer, ATCCTGTGGAACGCCATCA; and reverse primer, TTTCTTGAGGTCGGCGATCT, resulting in an amplicon size of 65 bp. The primer set used for mIGFBP-3 was: forward primer, GCAGGCAGCCTAAGCACCTA; and reverse primer, TGCTCCTCCTCGGACTCACT, resulting in an amplicon size of 70 bp. The primer set used for mGHR was: forward primer, CGGGTGAGATCCAGACAACG; and reverse primer, CCTAAGATGGTGTTCACCTCC,

resulting in an amplicon size of 299 bp. The primer set used for mGHBP was: forward primer, CGGGTGAGATCCAGACAACG; and reverse primer, CTTGGGATGGCGGATCCTCT, resulting in an amplicon size of 186 bp.

Threshold cycles were defined as the number of PCR cycles at which the fluorescent signal reached a fixed threshold signal. PCR efficiencies for all primer sets were greater than 95%. Relative expression levels were determined according to the comparative method (31). All measured values are normalized to household gene expression. The value of the placebo group is set at 1, and the values of other groups are recalculated to that, resulting in artificial values.

Statistical analysis

Means were compared by one-way ANOVA, followed by pairwise comparisons with the least significant difference method. Results are given as the mean ± SEM, with n indicating the number of mice studied. $P < 0.05$ was considered significant.

Results

Body, liver, and kidney weights; food consumption; and blood glucose and serum insulin levels

After 7 d of hIGFBP-1 and/or GHRA administration (experiment 1), none of the above-mentioned parameters was significantly changed in any of the groups compared with the control values (Table 1). Similar results were obtained after 2 d of hIGFBP-1 and/or GHRA administration (experiments 2 and 3; data not shown).

Serum GH, total and free IGF-I, IGFBP-1, and IGFBP-3

Basal serum GH levels were initially measured in experiment 1. Because no significant differences between the groups were found (data not shown), circulating GH levels were measured before and 10 min after stimulation with ipamorelin (experiment 2; Fig. 2). Stimulated serum GH values were markedly elevated in both groups receiving hIGFBP-1 to levels significantly above the stimulated GH levels in mice receiving placebo or GHRA ($P < 0.001$).

After 2 d of hIGFBP-1 and/or GHRA administration, serum total IGF-I and IGFBP-3 levels were significantly increased in mice receiving hIGFBP-1 compared with those in all other groups (Fig. 3A). There were no significant differences in free IGF-I levels among the groups (Fig. 3A). Serum IGFBP-1 levels were increased in both groups receiving hIGFBP-1 (Fig. 3A). Changes in circulating IGF-I and IGFBP-3 levels among the groups after 7 d were similar to those after 2 d (data not shown).

IGF-I and IGFBP-1 levels in tissues

Hepatic and renal IGF-I and renal IGFBP-1 protein levels were measured after 2 d of the hIGFBP-1/GHRA regimen without ipamorelin administration (experiment 3). Hepatic and renal IGF-I protein contents were increased in mice injected with hIGFBP-1 compared with levels in all groups (Figs. 3B and 4A). Renal IGFBP-1 levels were significantly higher in both groups receiving hIGFBP-1 compared with other two groups (Fig. 4B).

Expression of GH/IGF system genes in liver and kidney

The expression of GHR, GHBP, IGF-I, IGFBP-1, and IGFBP-3 genes was measured after 2 d of GHRA and/or hIGFBP-1 administration without ipamorelin stimulation (experiment 3). Hepatic GHR mRNA levels were significantly increased in mice receiving GHRA compared with those in groups receiving placebo and hIGFBP-1/GHRA (Fig. 5). GHBP mRNA levels were decreased in livers of both groups receiving hIGFBP-1 compared with those in mice receiving placebo, and in the hIGFBP-1/GHRA group compared with the GHRA group (Fig. 5). The expression of IGF-I and IGFBP-3 was decreased in both groups receiving GHRA compared with control values and in the hIGFBP-1/GHRA group compared with mice receiving hIGFBP-1 only (Fig. 3C). IGF-I mRNA levels were significantly higher in livers of mice receiving hIGFBP-1 than those in mice receiving placebo (Fig. 3C). Hepatic IGFBP-1 mRNA levels were decreased in all groups compared with control values, reaching significance in mice receiving GHRA alone and in mice receiving hIGFBP-1 alone (Fig. 3C). Renal GHR and GHBP mRNA levels were increased in the hIGFBP-1/GHRA group compared with those in the placebo and GHRA groups (Fig. 5). The increase in the hIGFBP-1 group compared with the placebo group was statistically significant only for GHBP mRNA levels. The expression of IGF-I was down-regulated

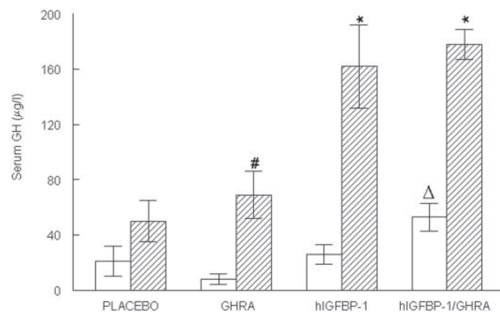

FIG. 2. Serum GH levels after 2 d, before (□) and 10 min after (▨) GH stimulation with ipamorelin in mice receiving placebo, GHRA, hIGFBP-1, or hIGFBP-1/GHRA. Values are the mean ± SEM (n = 7). *, $P < 0.001$ *vs.* placebo group (stimulated values); #, $P < 0.001$ *vs.* hIGFBP-1/GHRA group (stimulated values); Δ, $P < 0.05$ *vs.* all groups (basal values).

TABLE 1. Body weight (BW), liver weight (LW), kidney weight (KW), food consumption (FC), blood glucose levels (BGL), and serum insulin levels in adult female mice receiving placebo, GHRA, hIGFBP-1, or hIGFBP-1/GHRA for 7 d

	BW (g)	LW (mg)	KW (mg)	FC (g)	BGL (mM)	Insulin (μg/liter)
Placebo	18.3 ± 0.4	839 ± 72	120 ± 4	6.1 ± 0.4	6.7 ± 0.2	1.79 ± 0.34
GHRA	17.8 ± 0.3	916 ± 48	120 ± 3	6.2 ± 0.3	6.3 ± 0.2	2.04 ± 0.36
hIGFBP-1	18.1 ± 0.4	966 ± 65	121 ± 4	6.4 ± 0.4	6.0 ± 0.2	2.26 ± 0.29
hIGFBP-1/GHRA	18.0 ± 0.3	990 ± 38	118 ± 3	6.6 ± 0.4	6.4 ± 0.2	2.49 ± 0.44

Values are the mean ± SEM (n = 7).

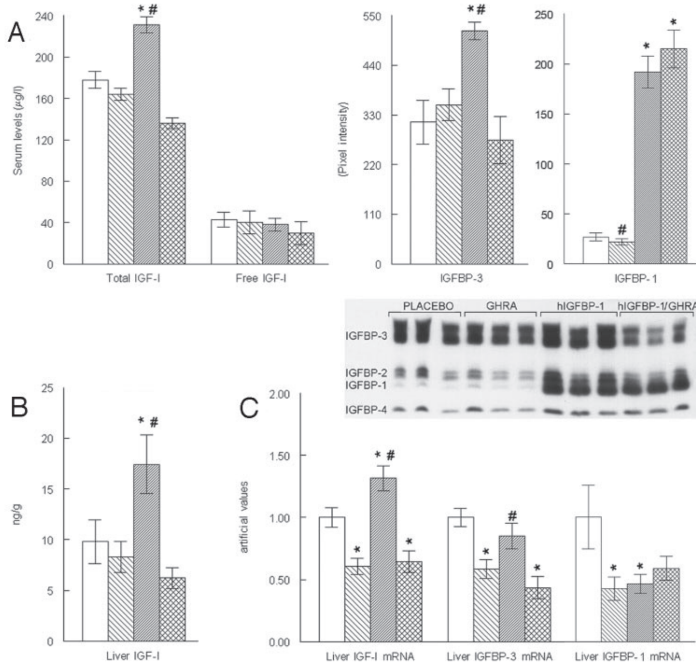

Fig. 3. Serum total IGF-I, free IGF-I, IGFBP-3, and IGFBP-1 (A), liver IGF-I (B), and liver IGF-I, IGFBP-3, and IGFBP-1 mRNA (C) levels after 2 d in mice receiving placebo (□), GHRA (▧), hIGFBP-1 (▨), or hIGFBP-1/GHRA (▩). Values are means ± SEM (n = 7). *, $P < 0.02$ *vs.* placebo group, #, $P < 0.01$ *vs.* hIGFBP-1/GHRA group. Insert: representative serum WLB, note that IGFBP-1 band consists of endogenous mIGFBP-1 and hIGFBP-1 in mice receiving hIGFBP-1 or hIGFBP-1/GHRA.

in kidneys of mice receiving hIGFBP-1/GHRA compared with placebo and hIGFBP-1 groups (Fig. 4A). Renal IGFBP-1 mRNA levels were significantly higher in both groups receiving hIGFBP-1 compared with controls (Fig. 4B).

Discussion

The major new finding of the present study is data supporting a direct, IGF-I-independent, stimulating effect of IGFBP-1 on GH secretion. The hIGFBP-1-induced increase in serum total IGF-I levels was abolished by GHRA coadministration, whereas the increase in serum GH was not affected. Furthermore, free IGF-I levels were unaffected by hIGFBP-1 administration. This suggests that the hIGFBP-1 induced increase in serum GH levels was not due to a IGF-I-dependent feedback mechanism.

Circulating IGF-I has an important feedback function within the somatotropic axis. Serum GH levels are increased in both liver-specific IGF-I knockout mice, also known as liver IGF-I-deficient (LID), consistent with the reduction in the inhibitory effect of circulating IGF-I on GH release from the somatotroph (32, 33). IGFBP-1 may affect GH secretion indirectly by lowering the free IGF-I level in the circulation, thereby activating a negative feedback. Several clinical investigations have suggested that IGFBP-1 is one of the most important regulators of IGF-I *in vivo* (34, 35). Transgenic (TG) mice with liver-specific expression of hIGFBP-1 have diminished serum total IGF-I levels and restricted postnatal growth, although serum GH is not altered (36–38). In these mice, the GH content of the pituitary was significantly reduced as a consequence of hIGFBP-1 overexpression, and exogenous hGH increased serum IGF-I and body weight. Insufficient GH production probably occurred due to a suppressed number of somatotrophs, with a resulting increased GH synthesis by the smaller somatotroph population reflecting an appropriate response of the hypothalamo-pituitary system to the sequestration of free IGF-I by excess IGFBP-1 (39). In concert with the observed reduction in body growth in IGFBP-1 TG mice, administration of hIGFBP-1 to Snell dwarf mice inhibited IGF-I-stimulated body growth, but stimulated kidney growth (22). Recombinant hIGFBP-1 administered to GH- or IGF-I-treated hypophysectomized rats reduces the effects of these growth stimulators on somatic growth (40). All of these studies indirectly indicate that IGFBP-1 is able to alter GH secretion via an IGF-I feedback mechanism.

The present study was designed to test the effects of excess IGFBP-1 on GH and IGF-I generation with and without peripheral blockade by GHRA. Although the administration of

GHRA alone did not have any effect on serum levels of IGF-I and IGFBP-3, it abolished the hIGFBP-1-induced increase in serum IGF-I and IGFBP-3 as well as their hepatic mRNA expressions. However, GHRA did not attenuate the hIGFBP-1-induced increase in ipamorelin-stimulated serum GH values; furthermore, there was no difference in free IGF-I serum levels between the groups. Hepatic GHR mRNA levels were increased in response to the functional peripheral blockade of the GHR by GHRA, as described previously (18). Up-regulation of the GHR concentration in liver microsomes was demonstrated in TG dwarf mice expressing GHRA (41). Coadministration of hIGFBP-1 normalized GHR gene expression in the liver. Taken together, these results suggest that hIGFBP-1 is directly responsible for the increased serum GH levels.

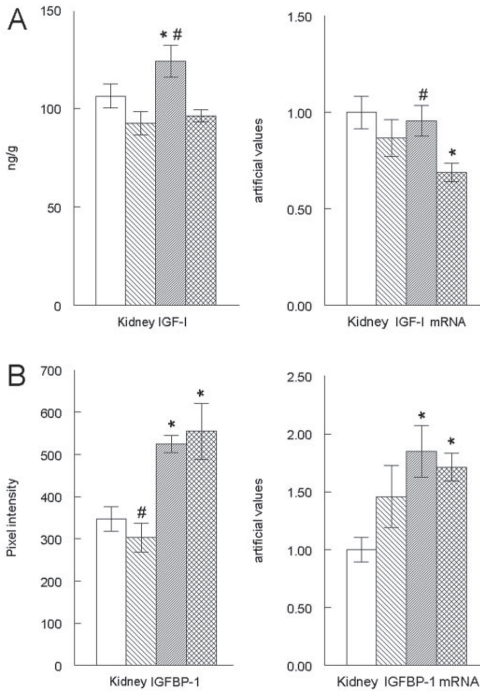

FIG. 4. Kidney IGF-I (A) and IGFBP-1 (B) protein and mRNA levels after 2 d in mice receiving placebo (□), GHRA (▧), hIGFBP-1 (▨), or hIGFBP-1/GHRA (▩). Values are the mean ± SEM (n = 7). *, $P < 0.01$ *vs.* placebo group; #, $P < 0.05$ *vs.* hIGFBP-1/GHRA group.

Another possibility is that IGFBP-1 down-regulates the bioactivity of the circulating IGF-I pool despite the unchanged levels of free IGF-I. Based on human studies, circulating levels of free IGF-I appear to be more closely related to changes in GH secretion than total IGF-I (42, 43). However, in mice, the role of circulating free IGF-I in controlling GH secretion remains less certain. In LID mice as well as in LID-ALS knockout mice, serum levels of free IGF-I are elevated despite markedly increased GH secretion and highly reduced levels of total IGF-I. In the present study we found normal levels of ultrafiltered free IGF-I after IGFBP-1 treatment. On the other hand, Lang *et al.* (44) recently published the results of study in which they observed a reduction in serum free IGF-I, as determined by ultrafiltration, after continuous iv infusion of purified human amniotic IGFBP-1. Whether it is the sc administration route, the sampling time (2.5 h after injection), or the IGFBP-1 dose that we chose that is responsible for the lack of reduction in free IGF-I after IGFBP-1 infusion remains unsolved. Because we used morning samples rather than longitudinal samples, we cannot fully exclude the possibility that free IGF-I levels were lower at other time points and therefore are indeed responsible for the increased GH secretion.

It is well known that serum IGFBP-1 levels are inversely related to changes in insulin secretion (13). However, GH is able to modulate the dominant insulin regulation of IGFBP-1, mostly by down-regulation of IGFBP-1 expression (13). In the present study GHRA administration decreased IGFBP-1 gene expression in the liver, although it did not alter IGFBP-1 serum levels. Furthermore, insulin levels were not affected by any of the treatments.

The renal IGF-I content was increased after hIGFBP-1 administration and was normalized by GHRA coadministra-

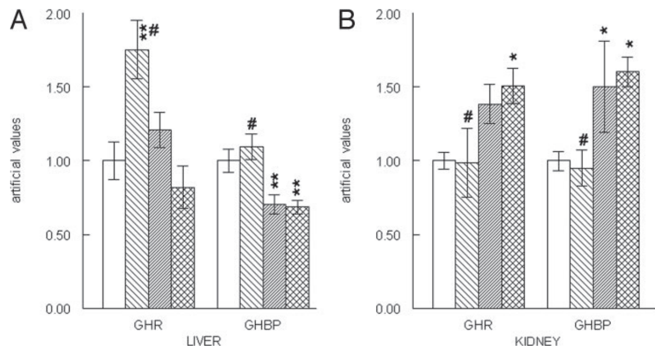

FIG. 5. GHR and GHBP mRNA in liver (A) and kidney (B) in mice receiving placebo (□), GHRA (▧), hIGFBP-1 (▨), or hIGFBP-1/GHRA (▩). Values are the mean ± SEM (n = 7). **, $P < 0.01$; *, $P < 0.05$ *vs.* placebo group. #, $P < 0.02$ *vs.* hIGFBP-1/GHRA group.

tion, reflecting the changes seen in serum and liver. However, renal IGF-I mRNA levels were unchanged after hIGFBP-1 injection (unlike hepatic IGF-I levels), suggesting that renal IGF-I accumulation is due to sequestration of circulating IGF-I, rather than to local synthesis. It is possible that IGFBP-1 traps IGF-I in the kidney. Accordingly, IGFBP-1 has been suggested to play a role in sequestering circulatory IGF-I, leading to the increased renal IGF-I content preceding kidney enlargement seen in early experimental diabetes in rodents (45). The administration of hIGFBP-1 to Snell dwarf mice resulted in kidney growth (22). Finally, it has been suggested that the well known inhibitory effect of a long-acting SS analog (octreotide) on the early increase in renal IGF-I concentration and renal size in diabetes may be mediated through a direct inhibitory effect on renal IGFBP-1 levels (46). Although GHRA administration significantly increased hepatic GHR mRNA levels, renal GHR and GHBP mRNA levels did not change significantly. These results, indicating that the regulation of mouse GHR expression is tissue specific, are in agreement with those of other studies examining renal GHR/GHBP mRNA levels (18, 21, 47, 48).

In summary, our data support a direct stimulating effect of IGFBP-1 on serum GH levels, suggesting that IGFBP-1 is involved in the complex network of feedback mechanisms in the regulation of GH secretion. Additional studies are warranted to elucidate the complex nature of the regulation of GH secretion and the specific role of IGFBP-1.

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Chapter 7

General Discussion

Introduction

The GH/IGF axis regulates somatic growth and cellular proliferation *in vitro* and *in vivo* through a complex system comprising GHR, GHBP, IGFs, IGF receptors and IGFBPs. IGF-I is a multifunctional polypeptide produced under the influence of pituitary GH (1, 2). The liver produces most of the circulating IGF-I although physiologically important autocrine and paracrine production occurs within other tissues. IGFs are potent mitogenic agents whose actions are determined by the availability of free IGFs to interact with IGF receptors. The level of free IGF in a system is modulated by rates of IGF production and clearance and the degree of binding to IGFBPs. IGFBPs not only regulate IGF bioavailability and action but also appear to mediate IGF-independent actions including regulation of cell growth and apoptosis (3, 4). IGFBPs are produced by a variety of different tissues and each tissue has specific levels of IGFBPs.

GH/IGF axis plays a major role in the mouse kidney physiology

GHR, GHBP and all members of IGF system are expressed in the mouse kidney (5-9). Alterations in GH/IGF axis, both locally in the kidney and in the circulation, have been linked to kidney enlargement in experimental mouse models, as described in detail in **Chapter 2**. Kidney enlargement is considered to be very important early step ultimately leading to renal failure. The available treatments for chronic renal failure, being either dialysis or kidney transplantation, make end-stage renal disease (ESRD) one of the most expensive diseases to treat in the developed world (10). The knowledge about early renal changes obtained from experimental models, is expected to facilitate the development of new strategies to abolish the decline of renal function at an early stage of the disease and possibly to restore the renal structure. The studies of the pathogenesis of the diabetic nephropathy, including the studies of involvement of GH/IGF system in renal physiology and pathophysiology, might also point out diagnostic markers for early prediction of the development of diabetic nephropathy (11).

1. Is kidney enlargement GH-dependent?

Different experimental approaches have been used to answer this question. Obvious choices would be models that either lack GH or overexpress GH. Hypophysectomy, surgical ablation of the pituitary gland in which GH is synthesized, has been one of the widely used approaches to investigate biological consequences of severe GH deficiency. Since the pituitary is the main regulator of the endocrine system, its removal results in gonadal, adrenal and thyroid gland deficiencies (12). This severe hormonal depletion alters metabolic parameters and affects the expression of genes involved in a variety of cellular functions (13). Snell dwarf mice, another model of GH deficiency, have a defect in the production of not only GH and IGF-I, but also prolactin and thyroid hormones (14, 15). Both of these models, with too many intrinsic variables, are not suitable for studying the effect of GH on kidney growth. Long-acting somatostatin (SS) analogues, such as octreotide and lantreotide, that suppress GH secretion from the anterior pituitary have proven useful in study of

GH/IGF axis (16, 17). Recently, a series of highly specific growth hormone receptor antagonists (GHRAs) has been developed for potential therapeutic use in various pathophysiological conditions including type 1 diabetes (18). This molecule competes with endogenous GH for the GHR and prevents GHR dimerisation, blocking peripheral GH action at the receptor level (**Chapters 4-6**).

To study the effect of GHRA in mice, we first established a dose dependency of the GHR blockade within a dose range 0-10 mg/kg/day (5, **Chapter 4**). No differences were observed in the groups with respect to body weight, food consumption or blood glucose. However, a dose-dependent decrease was observed in circulating IGF-I levels and in hepatic and renal IGF-I levels at the highest doses. The dose of 1.25 mg/kg/day did not affect the circulatory IGF-system levels and was previously shown to have significant renal effects (**Chapter 4**, 19, 20). This means that at this dose GH action in the kidney can be suppressed without influencing the circulatory IGF-system. Therefore, we used this concentration of GHRA to test involvement of GH in HP diet-induced renal hypertrophy (9, **Chapter 5**).

Blunted CRG in response to nephrectomy in hypophysectomized rats (21, 22), as well as prevention of CRG by octreotide in rats (23) and by GHRA in mice (19) give strong evidence that nephrectomy-induced renal growth is GH-dependent in rodents, at least in adults. Although CRG following nephrectomy seems to be GH-independent in adult female rats, in adult female mice it was shown to be GH-dependent (19, 24, 25). GHRA abolished not only nephrectomy-induced renal and glomerular hypertrophy but also renal IGF-I accumulation without affecting circulating levels of GH and IGF-I, presumably by blocking kidney GHRs (19).

Similarly, diabetes-induced renal growth, rise in renal IGF-I and the increase in UAE, are reduced in GH-deficient diabetic dwarf rats (26-29), and prevented by either long-acting SS analogue or GHRA treatment (17, 20, 23, 30-32) indicating that diabetes-induced kidney growth is also GH-dependent.

In case of HP-induced renal growth, GH-dependency was not clearly established. Rats fed HP diets demonstrated increased IGF-I paralleled by increase in renal weight, DNA synthesis, and mTAL length (33). Similar changes were seen in GH-deficient dwarf rats in response to HP diets, suggesting GH-independent renal growth (33). In rats with partial nephrectomy, HP diets even further increase renal weight and renal IGF-I content (34, 35). However, dietary protein content does not influence renal hypertrophy and renal IGF-I levels in GH-deficient dwarf rats, suggesting that GH mediates renotrophic effects of HP-diet (34, 35).

In pursuit of a unifying mechanism, in **Chapter 5** we investigated GH-dependency of renal enlargement induced by HP diet. We examined the effect of a newly developed GHRA on renal growth and renal GH/IGF-system expression in HP-fed mice. GHRA treatment neither abolished the HP-induced renal hypertrophy nor modified increased renal IGF-I, giving strong evidence that HP-induced renal enlargement, unlike nephrectomy- and diabetes- induced renal growth, is GH-independent.

2. IGF-I accumulates in enlarged kidney

IGF-I is a prime candidate for the mediation of renal growth. Systemic delivery of IGF-I (36-38) causing increased renal function, IGF displacers (39, 40) and

overexpression of IGF-I in TG mice (41-43) all lead to renal and glomerular hypertrophy. On the contrary, lack of IGF-I results in smaller kidneys with reduced number of nephrons and smaller glomeruli (44-46). It is not surprising then that renal IGF-I accumulation is a common event in all models of kidney growth both in rats and mice (**Chapter 2**). We confirmed this finding in both diabetes and HP-diet mouse models of kidney growth (**Chapters 3 and 5**). However, the mechanism of renal IGF-I accumulation in these models is still unknown. Following the onset of diabetes, IGF-I accumulates in the hypertrophying kidney despite unchanged/reduced renal IGF-I mRNA levels indicating that IGF-I is taken up from the circulation rather than produced locally (31, 47-49).

3. What is the role of IGFBP-1 in renal enlargement?

IGFBP-1, elevated in diabetic serum, is considered a strong candidate both in sequestering circulating IGF-I in the kidney and possibly in mediating kidney growth through IGF-independent actions (48, 50-52). Both long-acting SS analogue and GHRA abolish not only renal IGF-I accumulation, renal/glomerular hypertrophy and increased UAE (17, 20, 23, 30-32), but also early rise in renal IGFBP-1 mRNA in diabetic rodents (31, 53).

In normal mice, renal IGF-I content was increased after human (h)IGFBP-1 administration and normalised by GHRA co-administration, reflecting the changes seen in serum and the liver (**Chapter 6**). Renal IGF-I mRNA levels were unchanged after hIGFBP-1 injection (unlike hepatic IGF-I levels), suggesting that renal IGF-I accumulation is due to sequestration of circulating IGF-I rather than local synthesis. However, increased renal IGF-I levels after hIGFBP-1 administration, are not reflected on kidney weight in normal mice (**Chapters 3 and 6**).

The role of IGFBP-1 and IGF-I in the initial hypertrophy of the kidney was previously investigated in pituitary deficient Snell dwarf mice, expressing a disrupted GH/IGF-I axis (38). hIGF-I, hIGFBP-1, and a pre-equilibrated combination of equimolar amounts of hIGF-I and hIGFBP-1 were administered s.c. during 4 weeks. Administration of IGF-I alone induced a significant increase in body length (108% of control) and weight (112%) as well as an increase in kidney weight (124%). IGFBP-1 alone induced a significant increase in weight of the kidneys (152%). hIGFBP-1 co-administered with IGF-I inhibited the stimulating effects of IGF-I on body length and weight, but stimulated the growth promoting effect of IGF-I on the kidneys. The effect of IGFBP-1 plus IGF-I on kidney weight was not significantly greater than the effect of IGFBP-1 alone, suggesting a major role of IGFBP-1 in kidney enlargement in Snell dwarf mice (38).

To test the hypothesis that IGFBP-1 captures IGF-I in the enlarged diabetic kidney, we administered purified hIGFBP-1 to non-diabetic and diabetic mice (**Chapter 3**). We used similarly prepared hIGFBP-1 as the one administered to Snell dwarf mice, a comparable dose with respect to body weight and the same administration route, i.e. 3 daily subcutaneous injections. Surprisingly, renal IGF-I content was decreased in diabetic mice after hIGFBP-1 administration. The possibility that IGF-I excaped extraction due to binding to IGFBPs (especially IGFBP-1 present in excess) is excluded as the same extraction procedure was employed for all the groups and administration of hIGFBP-1 increased extractable renal IGF-I in control mice. As

serum IGF-I levels also showed differential response to hIGFBP-1 administration, being unchanged in control but decreased in diabetic animals, the difference in renal IGF-I accumulation could be due to lower serum IGF-I levels combined with very high serum IGFBP-1 levels, leading to reduced renal IGF-I uptake in diabetic mice.

Although increased levels of IGFBP-1 were detected in injected mice, both in serum and kidney, hIGFBP-1 had no further effect on initial kidney weight increase and albuminuria in STZ-diabetic mice. Furthermore, unchanged levels of renal IGFBP-1 in the diabetic mouse, along with unchanged serum IGFBP-1 levels, suggest that IGFBP-1 does not play a major role in kidney enlargement and renal IGF-I accumulation in STZ-diabetic mouse model. However it can not be excluded that a possible transient increase in renal IGFBP-1 at a time point before day 20, when the samples were taken, would play a role in the renal IGF-I accumulation seen in the diabetic kidney. Also it is possible that IGFBP-1 plays a role in later renal changes. hIGFBP-1 TG mice, displaying permanent, though moderate elevation of plasma IGFBP-1 levels develop glomerulosclerosis although glomerular hypertrophy is not seen (54, 55). Homozygous animals develop glomerulosclerosis at 3 months of age, followed by increased UAE at 8 months of age (55). Heterozygous animals, displaying nearly 5 times lower serum IGFBP-1 levels than homozygous mice develop glomerulosclerosis at 8 months of age (55). Although comparison with our data is difficult, these results stress the importance of IGFBP-1 dose and duration of administration/overexpression.

Renal expression of IGFBPs was generally unchanged or decreased in the STZ-diabetic mouse (**Chapter 3,** Table1). This is in contrast with studies reporting increased protein and mRNA levels of IGFBP-1 to –3 in STZ-diabetic rat (50, 56) and NOD mouse kidney (31, 32). However, it is in agreement with studies describing a decrease in renal IGFBP-3 to -5 mRNA levels, both in rat and mouse (48, 49, 57).

Our results suggest that IGF-I is captured in the kidney by mechanism not involving IGFBPs. However, further studies are needed to describe the alterations in IGFBPs in the diabetic kidney over time and to determine their pathophysiological importance.

4. Feedback within the GH/IGF axis

The classical endocrine effect of pituitary-secreted GH is the induction of IGF-I synthesis in various organs. The liver is believed to be the major source of circulating IGF-I which in turn is a negative feedback signal on GH secretion (58). The precise involvement of IGFBPs in regulation of GH secretion is at present unknown. They could be involved directly or indirectly through modifying free IGF-I. In **Chapter 6**, we explored the potential influence of IGFBP-1 on GH secretion by administering hIGFBP-1 and/or GHRA to mice. Our findings suggesting direct involvement of IGFBP-1 in GH stimulation break in upon the generally accepted ‘pecking order’ of the GH-IGF-IGFBP system where GH is believed to regulate IGF-I expression and the IGFBPs contribution to the system is thought to be solely in influencing IGF-I-IGF receptor binding. This is a totally new interpretation of the scientific data obtained in this research field.

Future directions

IGF-independent actions of the IGFBPs

The six high affinity IGFBPs originally seen as passive transport proteins for IGFs in the circulation, are increasingly recognised as active players in the circulation, extracellular environment and even inside the cell, through not only IGF-dependent but also IGF-independent mechanisms (59-61). Although our knowledge about the role and regulation of this multifunctional family of proteins increased over the years, much remains to be learned about their IGF-independent interaction with signalling pathways involved in proliferation, differentiation and apoptosis of various cell types. Recently reported intracellular (nuclear) actions of IGFBP-3 (62) open a new field of exploring the presence of other IGFBPs in the nucleus and their involvement in transcriptional events. Investigation of these new interactions could reveal new targets for treatment of kidney disease.

Interplay of GH/IGF system and other growth factor/cytokine systems

The GH/IGF system is only one of the many growth factor and cytokine systems expressed in kidney. These systems affect complex processes involved in renal physiology in an orchestrated manner. Therefore, renal pathology is believed to be multifactorial and experimental data accumulated so far suggest involvement of various growth factors and cytokines through complex endocrine and paracrine mechanisms.

Increasing number of studies report evidence for a prominent role of number of other growth factors and cytokines in diabetic complications. Besides IGF-I, factors that may be important in the development of early diabetic glomerulopathy include transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) (63-66). Numerous *in vivo* and *in vitro* studies suggest that TGF-β plays an important role in the accumulation of extracellular matrix observed during the course of diabetic nephropathy (65, 67). CTGF was suggested to act downstream of TGF-β and hepatocyte growth factor (HGF) possibly contributing to chronic tubulointerstitial fibrosis in diabetic nephropathy (68). Recently it was demonstrated that IGFBP-3 can bind to TGF-β binding protein-1 (LTBP-1), providing a potential mechanism whereby IGFBP-3 can interact with the TGF-β system (69). Glomerular hypertrophy caused by nephrectomy, diabetes or high-protein (HP) diet is VEGF-dependent (70-72). However, renal enlargement and increase in renal extractable IGF-I protein were not affected by administration of a VEGF antibody (70-72) indicating VEGF to be a downstream mediator of IGF-I, as previously suggested in diabetic retinopathy (73). Recent data obtained in human cells demonstrating that IGF-I induces VEGF gene expression and protein secretion in mesangial cells, suggest universal mechanism (74). Future studies investigating GH/IGF axis in renal physiology and pathophysiology are expected to further clarify the multiple interactions this system has with other growth factors, opening the way to development of new strategies in the prevention and/or treatment of renal diseases.

New technologies

Geneticaly modified mice overexpressing or exibiting lack of certain protein have been invaluable tools in studying the role of GH/IGF axis in kidney biology (**Chapter 2**). Further studies of existing mouse lines under various conditions, simultaneous modification of two or more members of the GH/IGF axis as well as temporal or tissue-specific refinement of genetic modification may give more clues.

Although many genes with important function in kidney pathophysiology have been described, still many more remain to be discovered. High-throughput methods of genomics such as DNA microarrays, used to simultaneously monitor several thousand different transcripts in a single experiment, promise to revolutionize our understanding of molecular mechanisms of kidney development and renal pathogenesis (75, 76). Microarray technology was already employed to define the gene expression profiles of the rodent kidney during development (77, 78), but also in specific models of renal disease such as in mice with experimentally induced proteinuria (79) and diabetic mice (80, 81). Efforts have been made to determine cell type specific gene expression profiles of murine renal cell lines representing e.g. podocytes, mesangial cells, proximal tubular cells, cortical collecting duct cells or medullary collecting duct cells (76). Microarray analysis could also be used to identify genes from other growth factor and cytokine systems interacting with GH/IGF system in models of renal disease. This powerful new technology offers means for a global analysis of the genetic basis of kidney biology and disease. Identification of genes previously not associated with kidney pathophysiology facilitates new approaches in molecular diagnostics and therapy design.

Parallel efforts have been made to determine protein expression levels and their posttranslational modifications, their sub-cellular localisation and interaction with other proteins (or DNA). Proteomics applications allow study of kidney disease pathogenesis at protein level, as well as development of new strategies for early diagnosis and treatment (82). These strategies assay diversity generated by posttranscriptional processing not provided by high-throughput mRNA screens.

Traditional ‘hypothesis-driven’ research to unravel renal disease pathogenesis, focusing on select candidate effector pathways such as IGF-I or TGF-β, slowly but steadily gives way for global-view, ‘technology-driven’, approach which seems to be more capable of obtaining insight into the complex and highly dynamic biological systems. Monitoring global cellular responses to disease or drug treatment by molecular profiling methods of genomics and proteomics, in specific nephron segments, results in unique fingerprints composed of molecular changes both in mRNA and protein expression levels that will aid identification of new markers of the renal disease and discovery of efficient treatments.

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Chapter 8

Summary

SUMMARY

The growth hormone (GH)/ insulin-like growth factor (IGF) axis constitutes a complex system of peptides consisting of GH and 2 IGFs (IGF-I and IGF-II), their respective receptors (GHR, IGF-IR, IGF-II/MP6R) and binding proteins (GHBP, IGFBP-1 to -6), present in the circulation, extracellular space and in most tissues. Being a major regulator of growth and metabolism, GH/IGF axis is also involved in renal development, growth, function and pathology.

IGFs are potent mitogenic agents whose actions are determined by the availability of free IGFs to interact with IGF receptors. The liver produces most of the circulating IGFs although physiologically important production occurs also within the kidney. Circulating and locally produced, renal IGFBPs regulate IGF bioavailability and action but also can mediate IGF-independent actions such as regulation of cell growth and apoptosis.

This thesis has focused on the GH/IGF axis role in renal changes following onset of diabetes and initiation of high-protein (HP) diet in mouse models.

Chapter 1 gives an overview of the GH/IGF axis members.

Chapter 2 deals with their expression in the mouse kidney and reviews current data obtained in rodent models, on the role of the GH/IGF axis in renal changes taking place after induction of diabetes, nephrectomy and initiation of HP diet.

Chapter 3 focuses on the contribution of the circulatory and the local renal GH/IGF system in early renal changes in experimental diabetes, i.e. increased kidney weight, increased glomerular volume and albuminuria. Following the onset of diabetes, IGF-I accumulates in the hypertrophying kidney despite unchanged/reduced renal IGF-I mRNA levels indicating that IGF-I is taken up from the circulation rather than produced locally. Hypothesis that IGFBP-1 captures circulating IGF-I in the diabetic kidney is not substantiated in streptozotocin (STZ)-diabetic mouse model.

Chapter 4 describes the role of GH in regulating circulating, hepatic and renal GH/IGF system in the mouse. A dose-response curve for a novel GHR antagonist (GHRA) is established. This molecule competes with endogenous GH for the GHR and prevents GHR dimerisation, blocking peripheral GH action at the receptor level.

In **Chapter 5** we examine the effect of the GHRA on renal growth and renal GH/IGF-system in HP-fed mice, to determine whether GH has a direct role in HP-induced renal growth. Unlike compensatory renal growth following nephrectomy and diabetic renal hypertrophy that are GH dependent, HP-induced renal enlargement is demonstrated to be GH independent.

Chapter 6 deals with regulation of GH secretion. Several studies indicate that IGFBP-1 is able to alter GH secretion via well-known IGF-I negative feedback loop, by reducing free IGF-I. We explore potential direct influence of IGFBP-1 on GH secretion and the effect of hIGFBP-1 administration on IGFBP-1 and IGF-I genes expression and their protein levels in the mouse liver and kidney.

In **Chapter 7** all data from the experimental chapters are combined and discussed in relation to current literature. Future studies are expected to clarify the multiple interactions the GH/IGF system has with other growth factors and cytokines in renal pathophysiology, opening the way for development of new medical treatments.

NEDERLANDSE SAMENVATTING

De groeihormoon (GH) / insuline-achtige groeifactor (IGF) as is samengesteld uit een systeem van peptides dat bestaat uit GH en twee IGF's (IGF-I en IGF-II), hun respectievelijke receptoren (GHR, IGF-IR, IGF-II/MP6R) en bindingseiwitten (GHBP, IGFBP-1 tot -6). Zij komen voor in de bloedsomloop, extracellulaire ruimte en in de meeste weefsels. Als een belangrijke regulator van groei en metabolisme, is de GH/IGF as ook betrokken bij de ontwikkeling van de nieren, hun groei, functie en pathologie.

IGF's zijn krachtige mitogene eiwitten waarvan de werking wordt bepaald door de beschikbaarheid van vrije IGF's die kunnen binden aan hun receptoren. De meeste circulerende IGF's worden geproduceerd door de lever, hoewel fysiologisch belangrijke productie ook plaatsvindt in de nieren. Circulerende en locaal geproduceerde IGFBP's uit de nier reguleren de beschikbaarheid van IGF en de activiteit, maar kunnen ook IGF-onafhankelijke acties beïnvloeden zoals regulatie van de celgroei en apoptose.

Dit proefschrift richt zich op de rol van de GH/IGF as in het optreden van veranderingen van de nier als gevolg van het verloop van diabetes en het overschakelen op een hoog eiwitdieet (eng. high-protein, HP) in muizenmodellen.

Hoofdstuk 1 geeft een overzicht van alle componenten van de GH/IGF as. **Hoofdstuk 2** gaat over hun expressie in de nier van de muis en behandelt ook de huidige onderzoeksgegevens uit knaagdiermodellen met betrekking tot de rol van de GH/IGF as in veranderingen van de nier die plaatsvinden na de inductie van diabetes, nefrectomie en de start van een HP dieet.

Hoofdstuk 3 richt zich op de bijdrage van het circulatoire en het locale GH/IGF systeem van de nier aan vroege veranderingen van dit orgaan tijdens experimentele diabetes, zoals toename in het orgaangewicht, toename in het glomerulaire volume en albuminurie. Tijdens het verloop van diabetes hoopt IGF-I zich op in de hypertrofiërende nier, ondanks onveranderde/ gereduceerde IGF-I mRNA niveaus van de nier die erop wijzen dat IGF-I wordt opgenomen uit de circulatie en niet locaal wordt geproduceerd. De hypothese dat IGFBP-1 het circulerende IGF-I wegvangt in de diabetische nier, kon niet worden onderbouwd in het streptozotocine (STZ)-diabetische muizenmodel.

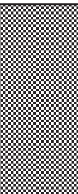
Hoofdstuk 4 beschrijft de rol van GH in de regulatie van circulerende GH/IGF eiwitten, in de nier en de lever van de muis. Een dosis/respons curve voor een nieuwe GHR antagonist (GHRA) werd opgezet. Dit molecuul gaat de concurrentie aan met endogeen GH voor de GHR en voorkomt GHR-dimerisatie door de perifere werking van GH te blokkeren op receptorniveau.

In **Hoofdstuk 5** bestuderen wij het effect van GHRA op de groei van de nier en op het GH/IGF-systeem van de nier in muizen op HP-dieet, om vast te stellen of GH een directe rol vervult in HP-geïnduceerde groei van de nier. In tegenstelling tot compensatoire groei van de nier als gevolg van nefrectomie en diabetische hypertrofie van de nier die GH-afhankelijk zijn, blijkt HP-geïnduceerde groei van de nier GH-onafhankelijk.

Hoofdstuk 6 gaat over regulatie van GH-secretie. Verschillende studies wijzen erop dat IGFBP-1 in staat is om de GH secretie te veranderen via een duidelijk beschreven IGF-I negatieve feedback mechanisme, door reductie van IGF-I. Wij

onderzoeken de mogelijke directe invloed van IGFBP-1 op de GH secretie en het effect van de toediening van hIGFBP-1 op de IGFBP-1 en IGF-I genexpressie en hun eiwitniveaus in de muizenlever en -nier.

In **Hoofdstuk 7** worden alle gegevens uit de experimentele hoofdstukken gecombineerd en besproken in relatie tot de huidige literatuur. Van toekomstige studies wordt verwacht dat zij helderheid verschaffen over de veelvoudige interacties van het GH/IGF systeem met andere groeifactoren en cytokines in de pathofysiologie van de nier, hiermee nieuwe wegen openend voor medische behandelingsmethoden.



PREGLED NA SRPSKOM

Osovina hormon rasta (engl. GH)/ insulinu-sličan faktor rasta (engl. IGF) obuhvata kompleksni sistem peptida koji čine hormon rasta, 2 insulinu-slična faktora rasta (engl. IGF-I and IGF-II), njihovi receptori (GHR, IGF-IR, IGF-II/MP6R) i vezujući proteini (GHBP, IGFBP-1 to -6), u krvotoku, vanćelijskom prostoru i u većini tkiva. Kao glavni regulator rasta i metabolizma, GH/IGF sistem je takođe uključen u razvoj, rast, normalnu funkciju i patologiju bubrega.

Insulinu-slični faktori rasta su moćni mitogeni čije dejstvo je određeno prisutnošću slobodne forme koja se vezuje za receptor. Najveća količina insulinu-sličnih faktora rasta prisutnih u krvotoku se proizvodi u jetri, ali se deo fiziološki bitne frakcije proizvodi takođe i u bubregu. Vezujući proteini (engl. IGFBPs) prisutni u krvotoku i proizvedeni lokalno u bubregu, regulišu dostupnost, a time i dejstvo insulinu-sličnih faktora rasta, a takođe mogu da posreduju i IGF-nezavisna dejstva uključujuci regulaciju ćelijskog rasta i apoptoze.

Ova teza se bavi ulogom GH/IGF osovine u promenama koje se dešavaju u bubregu miša nakon nastupanja šecerne bolesti (dijabetesa) i nakon davanja visoko-proteinske ishrane (engl. HP-diet).

Prvo poglavlje daje pregled članova GH/IGF sistema.

Drugo poglavlje se bavi njihovom zastupljenosti u bubregu miša, kao i pregledom najnovijih rezultata dobijenih u modelima glodara (miša i pacova) o ulozi GH/IGF osovine u promenama bubrega nakon odstranjivanja jednog bubrega ili dela bubrežnog tkiva, indukcije dijabetesa i davanja visoko-proteinske ishrane.

Treće poglavlje se bavi učešćem cirkulatornog i lokalnog bubrežnog GH/IGF sistema u ranim promenama bubrega u eksperimentalnom dijabetesu, kao sto su povećana težina bubrega, povećana zapremina glomerula i pojava albumina u urinu. Nakon nastupanja dijabetesa, IGF-I se nagomilava u uvećanom bubregu, uprkos nepromenjenim/smanjenim nivou bubrežne IGF-I mRNA. Ovo ukazujuje na to da je IGF-I verovatno preuzet iz cirkulacije, a ne proizveden u bubregu. Hipoteza da IGFBP-1 svojim vezivanjem zadržava IGF-I iz krvotoka u dijabetičnom bubregu nije potvrđena u streptozotocin (STZ)-dijabetičnom mišu.

Četvrto poglavlje opisuje istraživanje funkcije hormona rasta u regulaciji ekspresije GH/IGF sistema u krvotoku, jetri i bubregu miša. Utvrđena je dozno-zavisna kriva promena za novi antagonist receptora hormona rasta (GHRA). Ovaj molekul kompetira sa endogenim hormonom rasta za receptor hormona rasta (GHR) i sprečava dimerizaciju GHR, blokirajuci periferna dejstva hormona rasta na nivou receptora.

U **Petom poglavlju** smo ispitali efekat GHRA na rast bubrega i ekspresiju GH/IGF sistema u bubregu miševa koji su dobijali visoko-proteinsku ishranu, da bi odredili da li hormon rasta ima direktnu ulogu u rastu bubrega koji se javlja pri ovakvoj ishrani. Za razliku od kompenzatornog uvećanja bubrega koji se javlja nakon uklanjanja jednog bubrega ili dela bubrežnog tkiva i dijabetičnog uvećanja bubrega, koji su zavisni od hormona rasta, pokazali smo da je uvećanje bubrega koje se javlja usled visoko-proteinske ishrane nezavisno od hormona rasta.

Šesto poglavlje se bavi regulacijom lučenja hormona rasta. Nekoliko istraživanja je pokazalo da je IGFBP-1 u stanju da utiče na lučenje hormona rasta preko poznate negativne povratne sprege koja uključuje IGF-I, redukujući slobodnu formu ovog

faktora rasta. Ispitali smo potencijalno direktno dejstvo IGFBP-1 na lučenje hormona rasta i efekat administracije hIGFBP-1 na ekspresiju IGFBP-1 i IGF-I gena i nivo ovih proteina u jetri i bubregu miša.

U **Sedmom poglavlju** smo diskutovali rezultate svih eksperimentalnih poglavlja u odnosu na najnovije rezultate ostvarene na ovom polju. Buduća istraživanja bi trebalo da razjasne višestruke interakcije koje GH/IGF sistem ostvaruje sa različitim drugim faktorima rasta i citokinima u patofiziologiji bubrega, otvarajuci put za razvoj novih terapija.

Nature and Nature's laws lay hid in night: God said, "Let Tesla be", and all was light.

B. A. Behrend, speaking at the award of the Edison Medal to Nikola Tesla in 1917.

Curriculum vitae

Vesna Cingel-Ristić, born 24.7.1972. in Sremska Mitrovica, Yugoslavia (presently Serbia and Montenegro)

1987-1991 High School “Ivo Lola Ribar”, Sremska Mitrovica, Yugoslavia
Secondary School Degree, Specialisation: Science and Mathematics, Average mark 5 (out of 5)

1991-1996 Faculty of Biology, University of Belgrade, Yugoslavia
Bachelor of Science in Molecular Biology and Physiology
Average mark 9,52 (out of 10)
Project: Effect of the RelA Protein on the Resistance of *Escherichia Coli* K12 to the Antibiotic Novobiocin, carried out in the group of Prof Dr D. Savić at the Laboratory of Molecular Genetics of Prokaryotes at the Institute of Molecular Genetics and Genetic Engineering, Belgrade, Yugoslavia

1997-1998 International MSc in Biotechnology at De Monfort University, Leicester, United Kingdom and Hogeschool West-Brabant, Etten Leur, The Netherlands
Project: Heterologous expression of plant fructosyltransferases in yeast (*Saccharomyces cerevisiae*), carried out in the group of Prof Dr S. Smeekens at the Department of Molecular and Cell Biology, Molecular Genetics, University of Utrecht, the Netherlands

1999-2003 PhD student at the Laboratory of Pediatrics, Erasmus MC, Rotterdam, the Netherlands
Project: The GH/IGF axis in the mouse kidney, described in this thesis
Promotor: Prof Dr S. L.S. Drop

2004- postdoctoral fellow in the group of Dr J. Strouboulis at the Department of Cell Biology (Chairman: Prof Dr F. Grosveld), Erasmus MC, Rotterdam, the Netherlands
Project: Characterisation of YY1 function in erythropoiesis

Vesna is married to Dejan Ristić, a PhD student at the Department of Genetics, Erasmus MC. In 2001, they started a parallel, life-long project together. In June 2002, nearly 10 months long incubation at 37°C resulted in beautiful son Miloš.

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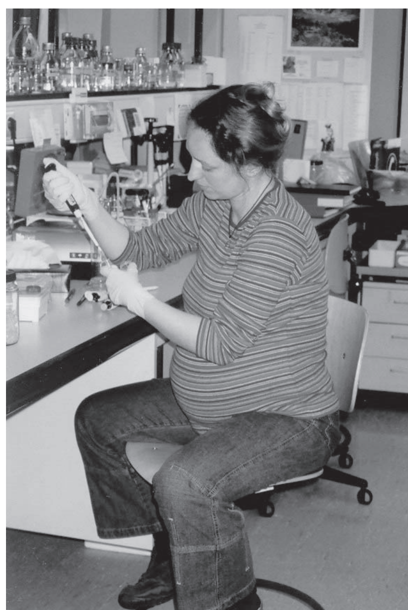


And in the end…

The love you take is equal to the love you make

The Beatles

Phew! Research that started in the last century (now far away January 1999) is finally getting its shape in this little thesis book. The journey was long, bumpy, with unexpected turns and the final destination was occasionally blurred. Nevertheless, here we are, at the end of this road and at the beginning of a new one. I would like to thank people who travelled with me. By shaping me into the person I am today, they helped me reach the place I am at now.

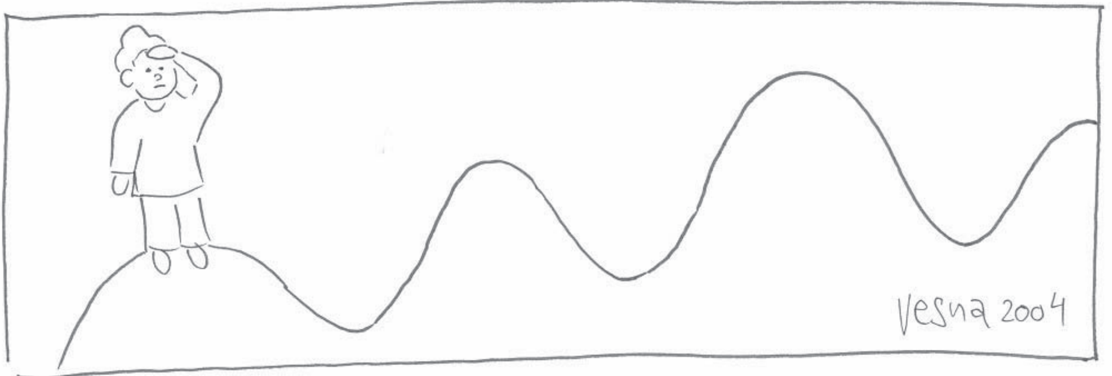
Sten, thank you for introducing me to the labyrinth of the GH/IGF world, buying my ticket for this exciting journey and seeing me through to the final destination. Han and Arlène, thank you for making the effort to keep me on the right path. Allan, most of all, thank you for holding the torch at the end of the long tunnel of GH, IGFs and kidney diseases. The time I spent working in your laboratory in Denmark provided me with great fun and I discovered that your incurable optimism is infectious.

I would also like to thank all the colleagues who were directly involved in the adventures described in this thesis and all the colleagues from Laboratory of Pediatrics (…Peter A, Ingrid R, Sandra, Edward, Maria, Barbara, Janneke, Dicky, Anita, Charlie, Rolien, Ytje, Peter H, Saskia, Debby, Marrieke, Wouter, Vishal, Marcel, Silvia, Ingrid L, Wim, Ad, Monique, Jules, Jessica, Amy, Karin, Ronald, Wendy, Pieter, Ada, Cora, Natasja, Melissa, Freek, John, Jeroen, Paul and especially my paranymph Theo…), who created good weather conditions along the way and frequently helped me overcome kinetosis. My ‘new’ colleagues from Cell Biology (…Javier, Tiago, Elena, Marianne, Katy, Patrick, Ernie, Edgar, John, Frank, Sanja, Arnie, Miyata, Ali, Farzin, Jurgen, Wouter, Erik, Helen…), thank you for a warm welcome. I am looking forward to our future adventures!

My friends from different corners of the world: Filipe, Laura, Eva, Sabrina and Pascal, Ikram, Dubi, Ksenija, Luca, Marrit, Mireille and Jos, Persa, Tanja, Barry, Elio, Nebojša, Peter, Bieke, Ivan…who better to understand my joy and troubles than you who had chosen similar paths?…, Jelena, Cristiano, Dragana, Han and Anne-Marie, Ozren and Seka, Hans, Joca and Marijana, Vita and Ivana, Rita and Scott, Saška, Še, Goran, Raj, Dick, Ramona…you enrich my life in so many different ways. Thank you for sharing experiences, thank you for love and support.

The power of words is limited but I would like at least to suggest the enormousness of my gratitude to my entire family, especially my Mum and Dad. Thank you for letting me choose my destinations and being with me all the way on every journey I took so far. My sister Miroslava, my sunshines Dejan and Miloš, I would like to mention once again that your patience and silent acceptance of my occasional (physical and mental) absences due to PhD matters was immensely appreciated.

And at the end I would like to thank problems that keep appearing along the way to wake up the slumbering voyager!


Vesna 2004

Nothing is perfec t