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Regulation of IGF-1 bioactivity by dietary hormones

"Impact of glucagon and insulin-induced hypoglycemia"

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Von Zeinab Sarem
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Gutachter

Prof. Dr. Florian F. Schweigert
Prof. Dr. med. Andreas F.H. Pfeiffer

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Summary

The relationship between nutrition and the development of chronic diseases including metabolic syndrome, diabetes mellitus, cancer and cardiovascular disease has been well studied. On the other hand, changes in the GH-IGF-1 axis in association with nutrition-related diseases have been reported. The interplay between GH, total IGF-1 and different inhibitory and stimulatory kinds of IGF-1 binding proteins (IGFBPs) results in IGF-1 bioactivity, the ability of IGF-1 to induce phosphorylation of its receptor and consequently its signaling. Moreover, IGF-1 bioactivity is sufficient to reflect any change in the GH-IGF-1 system. Accumulating evidence suggests that both of high protein diet, characterized by increased glucagon secretion, and insulin-induced hypoglycemia increase mortality rate and the mechanisms are unclear. However both of glucagon and insulin-induced hypoglycemia are potent stimuli of GH secretion. The aim of the current study was to identify the impact of glucagon and insulin-induced hypoglycemia on IGF-1 bioactivity as possible mechanisms. In a double-blind placebo-controlled study, glucagon was intramuscularly administrated in 13 type 1 diabetic patients (6 males /7 females; [BMI]: 24.8 ± 0.95 kg/m2), 11 obese subjects (OP; 5/ 6; 34.4 ± 1.7 kg/m2), and 13 healthy lean participants (LP; 6/ 7; 21.7 ± 0.6 kg/m2), whereas 12 obese subjects (OP; 6/ 6; 34.4 ± 1.7 kg/m2), and 13 healthy lean participants (LP; 6/ 7; 21.7 ± 0.6 kg/m2) performed insulin tolerance test in another double-blind placebo-controlled study and changes in GH, total IGF-1, IGF binding proteins (IGFBPs) and IGF-1 bioactivity, measured by the cell-based KIRA method, were investigated. In addition, the interaction between the metabolic hormones (glucagon and insulin) and the GH-IGF-1 system on the transcriptional level was studied using mouse primary hepatocytes. In this thesis, glucagon decreased IGF-1 bioactivity in humans independently of endogenous insulin levels, most likely through modulation of IGFBP-1 and-2 levels. The glucagon-induced reduction in IGF-1 bioactivity may represent a novel mechanism underlying the impact of glucagon on GH secretion and may explain the negative effect of high protein diet related to increased cardiovascular risk and mortality rate. In addition, insulin-induced hypoglycemia was correlated with a decrease in IGF-1 bioactivity through up-regulation of IGFBP-2. These results may refer to a possible and poorly explored mechanism explaining the strong association between hypoglycemia and increased cardiovascular mortality among diabetic patients.

Zusammenfassung

Der Zusammenhang zwischen Ernährung und der Entwicklung von chronischen Krankenheiten wie metabolischem Syndrom, Diabetes mellitus, Krebs und kardiovaskulären Erkrankungen wurde untersucht. Veränderungen der GH-IGF-1 Achse in Verbindung mit ernährungsbedingten Erkrankungen wurden früher beschrieben. Das Wechselspiel zwischen GH, gesamt IGF-1 und verschiedenen hemmenden und stimulierenden IGF-1 bindenden Proteinen (IGFBPs) bestimmt die IGF-1 Bioaktivität, die als die Fähigkeit von IGF-1 die Phosphorylierung von seinem Rezeptor und folglich seinem Signalsweg zu induzieren, identifiziert ist. Deshalb reicht die Messung der IGF-1 Bioaktivität aus, um Änderungen des GH-IGF-1 Systems darzustellen. Studien deuten darauf hin, dass proteinreiche Diät, gekennzeichnet durch erhöhte Glukagonsekretion, und Insulin-induzierte Hypoglykämie die Sterblichkeit erhöhen, und die Mechanismen sind unklar. Sowohl Glukagon als auch Insulin-induzierte Hypoglykämie stimulieren die GH Sekretion. Das Ziel der vorliegenden Studie war, die Wirkung von Glucagon und Insulin-induzierter Hypoglykämie auf die IGF-1 -Bioaktivität als mögliche Mechanismen zu characterizieren. In einer doppelblinden, Placebo-kontrollierten Studie wurde Glukagon intramuskulär 13 Patienten mit T1DM (6 Männer / 7 Frauen; [BMI] : 24,8 ± 0,95 kg / m2) , 11 übergewichtigen Teilnehmern (OP ; 5/6 ; 34,4 ± 1,7 kg / m2) und 13 gesunden schlanken Teilnehmern (LP ; 6/7 ; 21,7 ± 0,6 kg / m2) administriert. Zwölf übergewichtige Teilnehmer (OP ; 6/6 ; 34,4 ± 1,7 kg / m2) und 13 gesunde schlanke Teilnehmer (LP ; 6/7 ; 21,7 ± 0,6 kg / m2) führten Insulintoleranztests in einer weiteren doppelblinden, Plazebo- kontrollierten Studie durch. Änderungen des GH, gesamt-IGF-1, der IGF-bindenden Proteinen (IGFBPs) und der IGF-1-Bioaktivität wurden durch das zellbasierte KIRA-Verfahren gemessen. Außerdem wurde die Wechselwirkung zwischen den metabolischen Hormonen (Glucagon und Insulin) und GH-IGF-1-System auf der Transkriptionsebene mit primären Maus-Hepatozyten untersucht. In dieser Arbeit verringerte Glukagon die IGF-1-Bioaktivität bei den Menschen unabhängig von körpereigenen Insulinspiegeln, höchstwahrscheinlich durch Modulation des IGFBP-1 und -2. Die Glukagon-induzierte Reduktion der IGF-1-Bioaktivität stellt einen neuen Mechanismus der Wirkung von Glucagon auf die GH-Sekretion dar und kann als mögliche Erklärung für die negativen Auswirkungen der proteinreichen Diät im Zusammenhang auf das erhöhte kardiovaskuläre Risiko und die Mortalität vorgeschlagen werden.

Zusätzlich wurde die Insulin-induzierten Hypoglykämie eine Abnahme der IGF-1-Bioaktivität durch Hochregulierung von IGFBP-2 zugeordnet. Diese Ergebnisse können auf mögliche und wenig erforschte Mechanismen zur Erläuterung der starken Assoziation zwischen Hypoglykämie und erhöhter kardiovaskulärer Mortalität bei diabetischen Patienten beziehen .

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Abbreviation

GH	Growth hormone
IGF-1	Insulin like growth factor 1
IGF-2	Insulin like growth factor 2
kDa	Kilodalton
GHRH	Growth hormone releasing hormone
SST	Somatostatin
GHRHR	Growth hormone releasing hormone receptor
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element binding protein
DAG	Diacylglycerol
IT3	inositol triphosphate
GHS-R1a	Growth Hormone Secretagogue Receptor type 1a
GRF	Growth hormone releasing factor
GHR	Growth hormone receptor
JAK2	Janus- family Tyrosine Kinase 2
MAPK	Mitogen-activated protein kinase
IRS1	Insulin Receptor Substrate 1
PI3K	phosphatidylinositol -3- phosphate Kinase
SH2	Src homology 2
STAT	Signal transducer and activator of transcription

SOCS	Supressor of cytokine signaling
SHP1	SH2-containing protein tyrosine phosphatase-1
GHD	Growth hormone deficiency
LDL	Low density lipoprotein
IGF1R	Insulin like growth factor type 1 receptor
IGF2R	Insulin like growth factor type 2 receptor
IGFBPs	IGF binding proteins
AD	Alzheimer's disease
IR	Insulin receptor
pIGFBP-1	Highly phosphorylated IGFBP-1
LPIGFBP-1	Low phosphorylated IGFBP-1
npIGFBP-1	Non phosphorylated IGFBP-1
ALS	Acid-labile subunit
RXR	Retinoid X receptor
PAPP-A	Preganancy-associated plasma protein
PIP2	phosphotidylinositol (4, 5) diphosphate
PIP3	phosphotidylinositol (3,4,5) triphosphate
PDK1	phosphoinositide-dependent protein kinase-1
mTORC2	mammalian tor of rapamycin complex 2
FOXO1	Forkhead box O1
PEPECK	phoenolpyruvate carboxykinase
G6pase	glucose 6 phosphatase
FOXO3	Forkhead box O3

Bad	Bcl-2-antagonist of cell death
Bax	B cell lymphoma-associated protein X
TSC1	tuberous sclerosis complex 1
TSC2	tuberous sclerosis complex 2
Rheb	Ras homolog enriched in brain
Raptor	regulatory associated protein of mTOR
P70S6K1	P70 ribosomal protein S6 kinase 1
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
EIF-4E	eukaryotic translation initiation factor 4E
Grb2	growth factor receptor-bound protein 2
GEF	guanine nucleotide exchange factor
MEK	mitogen-activated protein kinase
ERK	extracellular signal regulated kinase
MDM2	Mouse double minute 2 homolog
TNFα	Tumor necrosis factor alpha
SOCS3	Supressor of cytokine signaling 3
GSK3	Glycogen synthase kinase 3
MuRF1	muscle RING finger-1
Nrf2	nuclear factor (erythroid-derived 2)-like 2
GCLC	Glutamate-cysteine ligase catalytic subunit
NQO1	NAD(P)H dehydrogenase (quinone) 1
HMOX1	Heme oxygenase (decycling) 1
HOMA-IR	Homeostatic model assessment of insulin resistance
ELISA	Enzyme-linked immunoassay

RIA	Radioimmunoassay
DMSO	Dimethyl-sulfoxide
FCS	Fetal calf serum
HSA	Human serum albumin
BSA	Bovine serum albumin
HEK	Human embryonic kidney
KIRA	Kinase receptor activation assay
RT	Room temperature
KRB	Krebs ringer buffer
TBST	Tris-buffered saline-Tween
SDS-PAGE	sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
APS	Ammonium persulfate
TEMED	N, N, N', N'-tetramethylethylenediamine
qRT-PCR	quantitative real time-Polymerase chain reaction

1. Introduction

During evolution, our endocrine system has been developed, starting in insects with a kind of peptides called insulin-like peptides secreted from the brain and mediated various functions like growth, carbohydrate metabolism, fertility and life span. In mammals, this system is divided into two functional-separated complementary networks, growth hormone (GH) and insulin, which are responsible for growth and glucose homeostasis, respectively. Insulin-like growth factor proteins (IGF-1 and IGF-2 prenatally and IGF-1 postnatally), that are considered as downstream products of GH signaling and involved in glucose homeostasis, seem to be conserved in mammals linking the functions of GH and insulin together and mediating growth, reproduction and glucose metabolism. GH/IGF-1 axis and its involvement in many important physiological pathways will be discussed in details in the following sections.

1.1 Growth hormone

1.1.1 Growth hormone structure

Translation of hGH (human Growth Hormone) gene that is found in the q22-24 region of the chromosom 17 results in a 22 KDa polypeptide consisting of about 191 amino acids. hGH protein forms a three-dimensional four-helix complex in an up-up-down-down behavior with connecting loops between helices 1 & 2 and helices 3 & 4 that are longer than one between helices 2 & 3. Two disulfide bridges are responsible for this complex stabilization. One bridge connects C53 belonging the large crossover loop between helix 1 and helix 2 to C165 from helix 4, the other disulfide bridge connects C182 to C189 belonging to the helix 4 and the C-terminal small loop respectively (1).

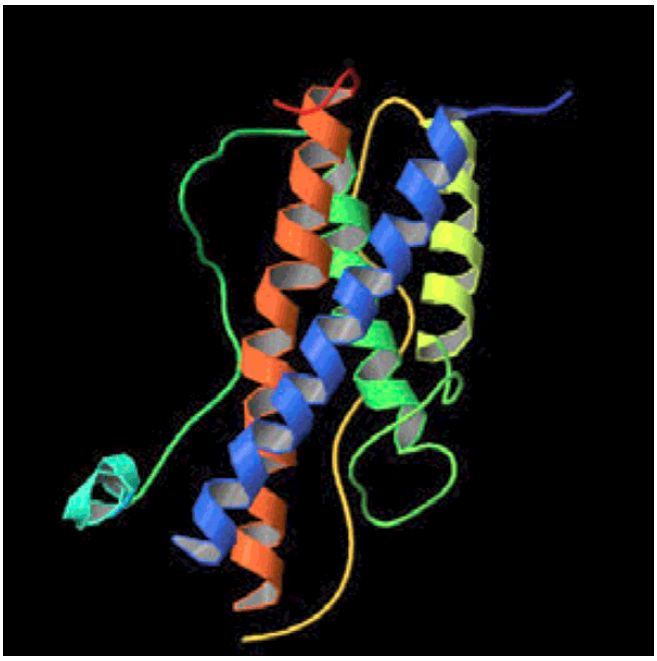

Figure 1: **Growth hormone structure** (2)

1.1.2 Biological regulation of growth hormone

GH secretion from cells called somatrophs, located in the anterior pituitary, is regulated by several different stimuli like gonadotropin-releasing hormone, corticotropin-releasing hormone, dopamine, ghrelin and thyrotropin-releasing hormone (3). The main regulator of its secretion is the interplay between growth hormone releasing hormone (GHRH) or somatocrinin and growth hormone inhibiting hormone or somatostatin (SST.) GHRH is mainly secreted in the arcuate nucleus, whereas SST is expressed in the periventricular nucleus of the hypothalamus (4;5). GHRH binds to GHRHR, a member of G-protein-coupled receptors and induces GH secretion through two different mechanisms. The first one is cyclic adenosine monophosphate (cAMP)-dependent pathway including GHRHR-binding and changes in its conformation, activation of α-subunit of the G-protein complex and stimulation of adenylyl cyclase leading to an increase in intracellular (cAMP). cAMP binds and activates the regulatory subunits of protein kinase A resulting in its translocation to the nucleus and phosphorylation and activation of cAMP response element binding protein (CREB) which enhances GH transcription by binding to the cAMP response element in the

promoter of GH gene. The other mechanism is phospholipase C-dependent which starts with GHRH-mediated simulation of phospholipase C through $\beta\gamma$ complex of heterotrimeric G-proteins and induction of diacylglycerol (DAG) and inositol triphosphate (IP3) which increase release of (intracellular calcium) Ca^{+2} from the endoplasmic reticulum resulting in increased secretion of GH. Conversely, somatostatin inhibits GH secretion via activation of tyrosine phosphatase that mediates the inhibition of the above mentioned calcium channels and adenylyl cyclase activity (6). The balance of these stimulating and inhibiting peptides which determines GH release is affected by other metabolic factors and hormones such as ghrelin, a gastric peptide secreted upon energy restriction. It induces GH release through its binding to the growth Hormone Secretagogue Receptor type 1a (GHS-R1a) by two mechanisms, one is by regulating GHRH and SST in hypothalamus and the other is by acting as a SST antagonist at the pituitary level (7;8). In opposite to ghrelin, leptin, a hormone secreted from white adipose tissues upon satiety and its blood levels correlate with body fat mass, modulates GH secretion through a direct regulation of growth hormone-releasing factor (GRF), somatostatin and alpha-melanocyte-stimulating hormone (9;10). Blood GH concentration is also influenced by various other nutritional, physiological and metabolic conditions such as gender. To date, it is known that increased androgen secretion during puberty as well as administration of either oestrogen or testosterone enhance plasma GH concentration (11;12). Age affects also GH secretion and it has been indicated that every decade of adult life is associated with a 14% decrease in GH secretion, following a peak during puberty. The change in blood GH level is not noticed only during the life but also during a day and the GH peak occurs about an hour after sleep during the slow wave sleep (13). Physical exercise as well as fasting and some amino acids such as leucine and arginine have stimulatory effects on GH (14-16). Conversely, glucose and fatty acids have inhibitory effects (17;18) . In addition to the previous factors, the circulating GH and its second mediator, IGF-1, can cross the brain-blood barrier, where they play an important role in regulating GH by a negative feedback mechanism on hypothalamus and pituitary (19;20).

1.1.3 Growth hormone functions

GH exerts its biological effects by binding to the extracellular domain of its receptor. The first step of GH signaling pathway occurs when a single active GH binds to two GH receptor (GHR) molecules inducing dimerization of the receptor. The dimerization of GHR leads to tyrosyl phosphorylation and activation of both GHR and GHR-associated JAK2 (Janus- family Tyrosine Kinase 2). These molecular events activate many effective downstream molecules such as MAPK (Mitogen-Activated Protein Kinases), IRS1 (Insulin Receptor Substrate 1), PI3K (phosphatidylinositol -3- phosphate Kinase), DAG, Protein kinase C and Ca^{+2} which are responsible for various GH functions like proliferation, enzymatic and transcriptional actions and involved in growth and metabolism (21). In addition to the above mentioned signaling pathway, the activation of JAK2 creates binding sites for the Src homology 2 (SH2) domains of the STAT (Signal Transducer and Activator of Transcription) proteins 1, 3, 5A and 5B resulting in their dimerization, nuclear localization and binding to specific regulatory sequences and increasing the rate of transcription of various genes as IGF-1 gene (22)**.** Moreover, a family of cytokine-inducible genes called SOCS (Supressor of Cytokine Signaling) and SHP1 (SH2-containing Protein Tyrosine Phosphatase-1) regulates GHR/JAK2 signaling through inhibition of JAK2 kinase activity and de-phosphorylation of nuclear STAT proteins respectively. The biological effects of GH result either from the direct binding of GH to its receptor or from its stimulation of IGF-1 secretion from the liver and other tissues. To these in growth and metabolism-involved effects belong all of lipolysis, proliferation and differentiation of chondrocytes, proliferation and differentiation of myoblasts, amino acid uptake, protein synthesis, triglyceride breakdown and insulin secretion.

1.1.4 Growth hormone disorders

1.1.4.1 Growth hormone deficiency

Growth hormone deficiency is identified as a failure of pituitary gland to produce enough amounts of growth hormone, due a partial or a complete lack of growth hormone production and is characterized by decreased both of body size and length. It can be congenital due to mutations of genetics or can be an acquired condition caused by many reasons such as brain infections or injury, brain tumors, decreased oxygen at birth or abnormalities of growth hormone receptors. Children with growth hormone deficiency may grow normally until the second or third year then start to grow slowly less than two inches per year in comparison with the normal children who grow at least two inches per year. Other children with growth hormone deficiency may grow slowly immediately after birth. They show also excess fat storage in the face and abdomen, as well as a low blood glucose concentration and a delayed puberty. Adults with GHD (Growth Hormone Deficiency) have increased body fat, decreased muscle and bone mass, impaired temperature regulation, depression, poor sleep and increased LDL-(low density lipoprotein) cholesterol.

1.1.4.2 Acromegaly

Acromegaly is a chronic condition caused by tumor-induced excess secretion of growth hormone. It must be differed between gigantism (excess growth hormone in prepubertal children) and acromegaly in adults. The most common symptoms of acromegaly are enlargement of hands and feet, swelling of soft tissues, excessive sweating, headaches and visual problems.

1.2 Insulin-like growth factor (IGF) system

IGF system consists of two ligands (IGF-1, IGF-2) which bind mainly to two cell-surface receptors, type 1 IGF receptor and type 2 IGF receptor (IGF1R, IGF2R), seven IGF binding proteins (IGFBPs) as well as IGFBP proteases.

1.2.1 Ligands

IGF-1 and IGF-2 are single-chain proteins consisting of 70 (IGF-1) and 67 (IGF-2) amino acids. Human IGF-1 and human IGF-2 share 62% of the sequences of each other (23). Despite this sequence similarity, IGF-1 and IGF-2 activate different signaling. It has been reported that mice with disturbance of IGF-1 or IGF-2 do not have the same birth weight as the wild-type mice (24). However, IGF-2 is not able to prevent the mental and growth retardation in patients with IGF-1 deficiency.

1.2.1.1 IGF-2

IGF-2 gene belongs to the imprinted genes, expressed in a parent-of-origin-specific manner and located on chromosome 11p15.5 (25). Some studies referred to the role of IGF-2 in fertility by increasing proliferation of granulosa cells and synthesis of estradiol and progesterone (26;27). Other studies found that activation of IGF-2 signaling contributes to treatment of diseases such as alzheimer's disease (AD) via enhancement of neurogenesis (28). However, although it is expressed in humans in different tissues throughout the life but it plays a more essential role in embryogenesis and fetal development (29). Therefore, IGF-2 is believed to be a fetal growth regulator promoting growth during gestation and regulating the size and exchange capacity of the placenta in all vertebrate species, comparable with IGF-1 which is considered to be an adult growth factor (30). Despite some reports referred that liver IGF-2 production is not influenced by GH in postnatal stage (31), others showed that liver IGF-2 production in teleost fishes is regulated, in addition to GH, by insulin and cortisol (32) and affected by fasting-refeeding state (33). However, how its secretion is regulated remains unknown.

1.2.1.2 IGF-1

IGF-1 is a 7.5 kDa 70-amino acid single-chain polypeptide with 49% sequence homology to insulin. At the beginning, it was called “somatomedin” and then subsequently identified as insulin-like growth factor 1, organized with three disulfide bridges that

facilitate its binding to its receptor. It is synthesized as an endocrine hormone mainly in the liver under the stimulation of GH and secreted in bloodstream, where it acts as a feedback regulator of GH secretion on the hypothalamus and pituitary gland levels (31). It differs from other hormones that it is also secreted in many extrahepatic tissues where it exerts its autocrine/paracrine effects. IGF-1 is produced from IGF-1 gene which consists of more than 6 exons and located in the chromosome 12 in humans and in chromosome 10 in mice (34).

1.2.2 IGF receptors

They form a family of transmembrane proteins that bind IGF-1, IGF-2 or both of them and consist of IGF1R, IGF2R and insulin receptor (IR). These tyrosine kinase receptors consist of two extracellular α-subunits which bind IGF and two β-subunits which have intracellular tyrosine kinase domain and linking together with disulfide bridges. The presence of a binding site for ATP in these receptors is responsible for their autophosphorylation after their binding to the corresponding ligands, induction of various intracellular signaling and phosphorylation of other cellular molecules. Overexpression of IGF1R is associated with more cell proliferation and carcinogenesis, while its depletion increases the resistance to oncogenic transformation (35;36). Because of its expression on many tumor cell types and its ability to decrease apoptosis and increase cell survival, it was an important target for tumor treatment (37). Using IGF1R antisense ribonucleic acid, dominant-negative truncated IGF1R or IGF1R neutralizing antibodies demonstrated the positive effects of IGF signaling inhibition on tumorigenesis (38-40). In addition, its critical role in inducing epithelial cell proliferation of mammary glands during pregnancy and lactation has been reported. IGF1R is the most important member of this family with a molecular weight of 320 kDa, 60% sequence homology of IR and a higher affinity to IGF-1 compared with IR. Because of the sequence similarity of IGF-1 and insulin and IGF1R and IR and the 100-fach less affinity of IGF-1 to IR, IGF-1 binding to a IGF1R-insulin receptor combination has been demonstrated. Moreover, IGF2R binds IGF-2 only and despite it mediates no intracellular signaling, it usually works as a clearance receptor preventing IGF-2 signaling.

1.2.3 Insulin-like growth factor binding protein (IGFBP) family

IGFBP family comprises 7 binding proteins (IGFBP-1 to IGFBP-7) that share similar structures and variable but high affinity to IGF-1 resulting in modulation of IGF-1 actions. All IGFBPs have a common conserved domain consisting of the N-terminus, IGF binding protein domain, and the C-terminus, thyroglobulin type-1 repeat, while the mid-region differs among them depending on the presence of proteolysis, phosphorylation and glycosylation sites. In addition to functioning as carrier proteins, prolonging the half-life time of IGFs, and preventing their binding to IGF receptors, they can act as modulators of different physiological processes, independent of IGFs (41). 80 % of the IGFs circulate in the blood forming a complex of 150 kDa consisting of one molecule of IGF-1 or IGF-2, a 85 kDa acid-labile subunit (ALS) and IGFBP-3, 20-25 % of the IGFs are bound to the other IGFBPs forming a complex of 50 kDa, while less than 1 % of IGFs are found in free form of 7.5 kDa (42).

1.2.3.1 GFB -1

IGFBP-1 is found in the liver, ovary, the amniotic fluid and the plasma. It is a 25 kDa protein and subject to serine phosphorylation at three different phosphorylation sites. Depending on the degree of phosphorylation, it is found in three isomers (highly phosphorylated IGFBP-1, pIGFBP-1, less phosphorylated IGFBP-1, Lp IGFBP-1 and non-phosphorylated IGFBP-1, np-IGFBP-1). Under normal physiological situations, IGFBP-1 is found circulating in the phosphorylated form that is able to bind IGF-1 and modulate its activity. It is regulated by a variety of stimuli like glucagon, exercise, thyroid hormones, growth hormone and glucocorticoids (43-46)**.** However, the main regulator of IGFBP-1 is insulin that inhibits IGFBP-1 synthesis and secretion from the liver. Circulating IGFBP-1 level is considered as an important predictor of insulin secretion and its decrease refers to hyperinsulinaemia or to long-term type 2 diabetes mellitus (47;48), whereas a high serum level of IGFBP-1 is associated with insulin resistance (49).

1.2.3.2 IGFBP-2

IGFBP-2 with a molecular weight of 36 kDa is the second most abundant circulating IGFBP after IGFBP-3. It exerts a higher binding affinity to IGF-2 than IGF-1 (42). Both stimulatory and inhibitory effects of IGFBP-2 on IGF-1 actions and positive and negative regulation of IGFBP-2 of tumor growth have been described, depending on tissue type and physiological conditions (41;50-52). Increased serum concentration of IGFBP-2 has been reported in non-islet cell tumor hypoglycemia and diabetes mellitus (53).

1.2.3.3 IGFBP-3

It is the major circulating binding protein for IGFs, binds 80-90% of all IGF-1 found in the blood. It is a 24-45 kDa glycoprotein secreted systematically from the liver and locally from other normal and cancer cell types. It exists in a 140-150 kDa ternary complex with IGF-1 or IGF-2 and with a 85 kDa glycoprotein, acid labile subunit (ALS). This complex is not able to pass the capillary barrier and its formation is inhibited by glycosaminoglycans. Growth hormone deficiency decreases blood IGFBP-3 concentration, while it increases in patients with acromegaly. In addition to its IGF-1-dependent effects, its direct modulation of cell growth and survival has been demonstrated. Interacting with nuclear elements like retinoid X receptor α (RXR) and cellular proteins like Alzheimer's survival protein, humanin, inducing apoptotic protein Bax and increasing cell sensitivity to ionising radiation through IGFBP-3 have been reported as possible mechanisms for IGFBP-3 - cell growth interaction (54;55). However, down-regulated expression of IGFBP-3 has been observed in many tumor cells and considered as a powerful diagnostic biomarker of cancer development (56).

1.2.3.4 GFB -4

Existing in two forms, non-glycosylated (24 kDa) and N-glycosylated (28 kDa), IGFBP-4 is the smallest IGF-binding protein. It binds IGF-1 and IGF-2 with the same affinity without any influence of the glycosylation state. It is secreted from the liver and other different cell types and tissues like adrenals and testis. However, it secretion is affected by

different nutritional and hormonal factors like caloric intake, vitamin D and para thyroid hormones and its ability to bind IGFs is regulated by its proteolysis trough different proteases like preganancy-associated plasma protein (PAPP-A) (57;58). In addition to its inhibition of IGF-1 pathway, IGF-independent actions of IGFBP-4 like stimulation of cardiomyocyte differentiation have been reported (59). Moreover, a tissue-dependent positive and negative association between IGFBP-4 expression and tumor growth has been suggested (60;61).

1.2.3.5 IGFBP-5

It is with a molecular weight of 29 kDa located like IGFBP-2 in chromosome 2. It is expressed in many prostate cell models and cell types like human fibroblasts, ovarian granulose cells and chondrocytes. It shows a low but similar binding affinity to IGF-1 and IGF-2 (62). In addition to the various reports indicating the inhibitory effect of IGFBP-5 on the actions of IGF-1, IGFBP-5 has been found to stimulate IGF-1 signaling in prostatic disease (62).

1.2.3.6 IGFBP-6

A 32 kDa protein found preferentially in cerebrorospinal fluid with a higher affinity to IGF-2 over IGF-1. It is found in O-glycosylated form that is required for its secretion, stability and translocation, but not for its binding to IGFs and inhibition of their actions. Its association with the development of diseases like type 1 diabetes mellitus has been reported (63).

1.2.3.7 IGFBP-7

It differs from the other six IGFBPs that it lacks the C-terminus and has a low affinity to IGFs but a high affinity to insulin. A lower IGFBP-7 expression has been observed in different cancer types like breast, prostate and colorectal cancer compared with normal cells. However, it seems to function as a powerful tumor suppressor (64;65).

1.2.4 IGF-1 bioactivity

In order to measure total circulating IGF-1, many immunoassays like ELISA, RIA ...have been developed. IGFBPs form complexes with IGFs, dissociate from them very slowly and give false results in these assays, therefore, techniques like acid-dependent size exclusion chromatography, extraction with solid-phase and acid-ethanol extraction have been used. Despite of the above mentioned extraction procedures, the remaining IGFBPs may still interfere with the used assay. Nevertheless, to measure IGF-1 efficiency with the presence of IGFBPs, various methods have been evaluated, including measurement of IGF-1-induced sulfate incorporation, DNA synthesis, RNA synthesis, protein synthesis and glucose uptake. Recently, an assay called kinase receptor activation assay (KIRA) that takes in consideration the inhibitory and stimulatory effects of IGFBPs on IGF-1 signaling, has been widely used (66;67).

1.2.5 IGF-1 signaling

Binding of IGF-1 to the extracellular α-subunit of its receptor, IR or IGF1R/IR leads to a conformational change in the receptor structure and to autophosphorylation of tyrosine residues in the intracellular β-subunit of this receptor. IRS1 binds to the phosphorylated receptor and the resultant activated IRS1 serves as a binding and activating site for the PI3kinase which phosphorylates phosphatidylinositol (4, 5) diphosphate (PIP2), a component of the cell membrane that is integrated into the two lipid layers of the cell membrane, and forms phosphatidylinositol (3,4,5) triphosphate (PIP3) which can also bind and activate AKT kinase or protein B kinase. Activation of protein kinase B demands its phosphorylation on threonine 308 by phosphoinositide-dependent protein kinase-1 (PDK1) as well as its phosphorylation on serine 473 by mammalian target of rapamycin complex 2 (mTORC2) (68;69)

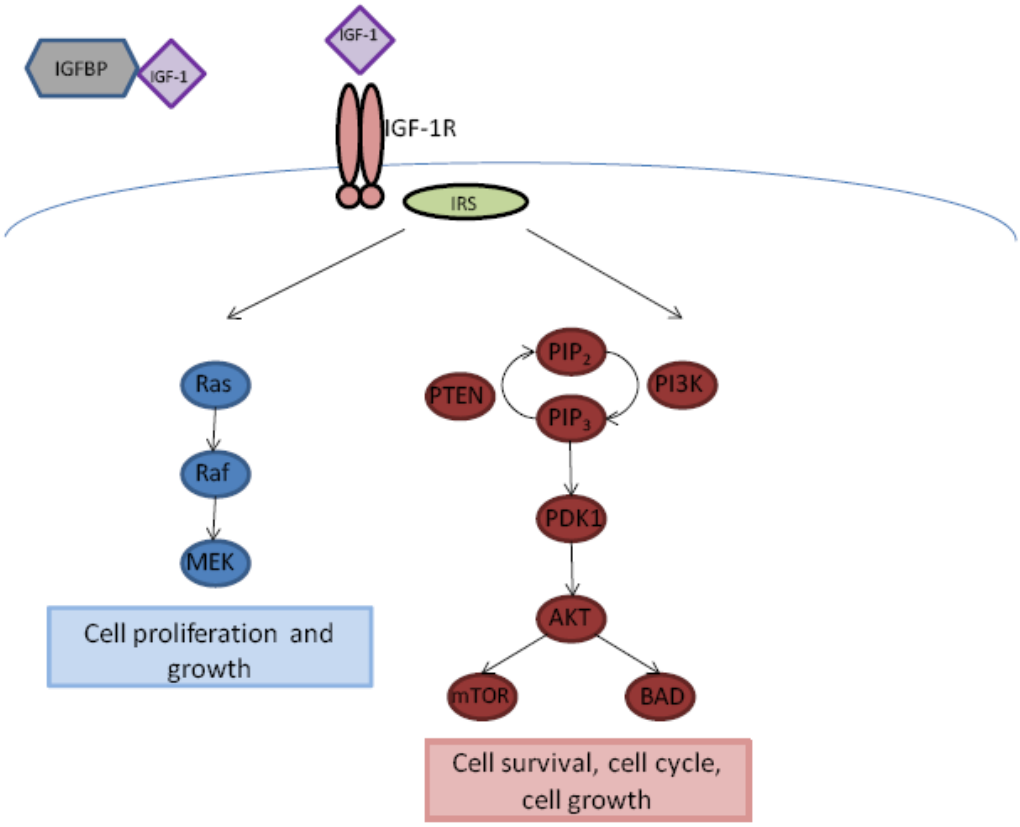

Figure 2: IGF-1 signaling pathway (70)

Activation of Akt is responsible for many metabolic processes by affecting many of other cellular proteins and nuclear transcription factors. One of them is the Forkhead box (FOX) family of nuclear transcriptional factors and its member forkhead box O1 (FOXO1). FOXO1 is important for transcription of gluconeogenic genes such as phoenolpyruvate carboxykinase (PEPCK) and glucose 6 phosphatase (G6Pase) in the liver as well as of genes mediating hepatic fatty acid oxidation and ketogenesis (71;72). When FOXO1 is phosphorylated by PKB/Akt on threonine 24, serine 319 and serine 256, it translocates from the nucleus to the cytoplasm limiting the transcription of target genes (73). Another member of FOX family affected by Akt phosphorylation is the pro-apoptotic forkhead box O3 (FOXO3) mediating the anti-apoptotic effects of insulin or IGF-1 signaling (74). The anti-apoptotic effects of Insulin and IGF-1 are mediated also by inactivation of other pro-apoptotic proteins such as Bcl-2-Antagonist of Cell Death (Bad) and B cell lymphoma-associated protein X (Bax) (75;76). The tuberous sclerosis complex TSC1/TSC2, another downstream of Akt phosphorylation, inhibits mTOR signaling by inactivation of a small G

protein called Ras homolog enriched in brain (Rheb). Mutations in the tumor suppressor genes TSC1 and TSC2 cause a human syndrome known as tuberous sclerosis complex syndrome and characterized by a development of harmatomas, skin rashes and mental retardation (77). Phosphorylation of Akt phosphorylates TSC2, a GTPase activating protein and a component of TSC1/TSC2 complex, and re-destributes this complex from the cell membrane to the cytosol dissociating the interaction between TSC2 and Rheb and leading to more accumulation of the active form of Rheb and to more activation of mTOR signaling (78).

There are two complexes of mTOR, one is called mTORC1 which consists of mTOR, regulatory associated protein of mTOR (Raptor), mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) and is inhibited by rapamycin. Its activation is mediated by Akt signaling through inactivation of TSC1/TSC2 complex. The most important downstream targets of mTORC1 are P70 ribosomal protein S6 kinase 1 (p70S6K1) which activates S6 Ribosomal protein and stimulates protein synthesis and the eukaryotic initiation factor 4E binding protein 1(4E-BP1) which after its phosphorylation by mTORC1 releases eukaryotic translation initiation factor 4E (eIF4E) allowing it to bind to mRNA and promote protein synthesis. mTORC1 is considered to be a nutrient, energy and redox sensor. The presence of growth hormones activates mTORc1 via Akt signaling and low levels of ATP under conditions of starvation reduce mTORC1 via AMPK-dependent activation of TSC2. IGF-1 is known to induce differentiation and proliferation and this effect is mediated by binding the small adaptor protein called growth factor receptor-bound protein 2 (Grb2) to the IRS proteins via SH2 domain. Activated Grb2 binds to the guanine nucleotide exchange factor (GEF) called SOS via SH3 domain which promotes the phosphorylation of Ras. The phosphorylation of Ras enables its recruitment to the plasma membrane where it activates a small G protein P21 Ras which binds and activates the serine/threonine kinase Raf. Activation of its downstream effectors as mitogen-activated kinase (MEKs) and extracellular signal regulated kinases (ERKs) leads to the translocation of ERK in the nucleus and phosphorylation of different transcription factors which are involved in differentiation and proliferation processes. Another pathway which is involved in IGF-1 effects is the apoptosis signaling. Studies have mentioned that IGF-1 through PI3kinase pathway increases P53 degradation and decrease P53 protein levels by induction of phosphorylated Mouse double minute 2 homolog (MDM2), an ubiquitin ligase, which

binds to P53 and inhibits its DNA binding decreasing the transcription of apoptosis-mediated genes (79).

1.2.6 IGF-1 concentration

The normal range for growth hormone is 1-9 ng/ml. However, a lower as well as a higher value may be normal because GH is secreted in pulses. IGF-1 level is used as a screening test for growth hormone diseases such as growth hormone deficiency, dwarfism, acromegaly and gigantism. IGF-1 concentration in the blood ranges between 10 and 1000 ng/ml and it is changed by age, gender, pubertal stage and nutritional state (80).

1.3 GH/IGF-1 axis and diseases

Aging is characterized by loss of muscle mass and impairment of its function increasing the risk for falls and fractures (81;82). Decreased GHR and IGF-1 mRNA and protein and increased tumor necrosis factor alpha (TNFα) and suppressor of cytokine signaling 3 (SOC-S3) mRNA in skeletal muscle biopsies from older male compared with younger ones suggest the role of GH/IGF-1 signaling in this disorder (83)**.** Through mTOR and Glycogen synthase kinase 3 (GSK3) and forkhead transcription factors, downstream effectors of Akt signaling, and reduction of E3 ubiquitin ligases, atrogin-1 and muscle RING finger-1 **(**MuRF1), IGF-1 induces protein synthesis and myocyte proliferation and increases muscle growth (84;85). Thus, decreased GH-dependent IGF-1 in parallel with aging could be responsible for the age-related muscle mass wasting.

IGF-1 is involved in bone generation and mineralization through activation of osteoblast proliferation and modulation of phosphate and 25-hydroxyvitamin D3 re-absorption at the kidney level (86)**.** Furthermore, GHR- and IGF-1-knockout mice show skeletal malformation, decreased mineralization and increased apoptosis which are reversed by administration of IGF-1 (86), therefore, decreased level of plasma IGF-1 contributes to the pathogenesis of osteoporosis. There is also an increased incidence about the relationship between low IGF-1 levels and increased cardiovascular mortality and heart failure (87). Many mechanisms are expected to mediate the positive effect of high IGF-1 levels as an increase in the expression of Mouse double minute 2 homolog (MDM2) and the resulting

decrease of P53-related genes such as Angiotensinogen, Bax and Ang type1 or an increase in the expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and Nrf2-related genes such as Glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H dehydrogenase (quinone) 1 (NQO1) and heme oxygenase (decycling) 1 (HMOX1) which are responsible for the antioxidant defenses in the vessels (88;89)**.** Low plasma levels of IGF-1 are also correlated with metabolic syndrome and its disorders such as hepatic steatosis, diabetes mellitus type 2, chronic kidney disease and hypertension (90-93)**.** It has been shown that IGF-1 contributes to glucose metabolism because the mutant disruption of IGF-1 leads to a severe insulin resistance which could be reversed by exogenous IGF-1 treatment (94). Studies with pancreatic β cells showed that IGF-1 knockout mice indicated a decreased insulin secretion suggesting the role of IGF-1 in insulin secretion (95). Schäfer *et al.* found that the elevated IGF-1 serum level is the reason for the protection of Berlin fat Mouse Inbred Line against diabetes (96).

1.4 IGF-1 and nutrition

Many metabolic indicators are used to evaluate nutritional status especially in hospital patients which may show evidence of malnutrition or even development of nutritional deficiency. These indicators should be applicable to assess the nutritional status before initiating of therapy and to evaluate the short-term response to the nutritional interventions. To these indicators belong the anthropometric determinations which show low response to the altered nutritional status and serum albumin and transferrin measurements which are also limited in monitoring of the short-term changes because of their long half-lives (97;98). Nitrogen balance, a difference between protein N intake and urinary urea N loss is also used, however, this index is not reliable in such conditions as severe burns or gastrointestinal diseases. Compared with the previous indicators, IGF-1 determinations are suggested to be more useful in assessing the nutritional status. Many in vivo and in vitro studies reported that the exposure of the liver to malnutrition leads to decreased secretion of IGF-1 (99). Other studies showed that IGF-1 blood concentration increased rapidly after nutritional support and this increase depended on the quality and quantity of food. Western diet which consists of grain-based food, sugars and dairy products is known to increase insulin/IGF-1 signaling because of its high

glycemic index and insulinotropic dairy (100). In contrast, dietary restriction and paleolithic diet which excludes the previous products decreases IGF-1. Some studies referred that the acute postprandial hyperinsulinemia following the increased intake of refined carbohydrates and the resulting changes in IGF-1 system is the main reason for the increased growth in groups such as Eskimo (101).

2. Aim of study

The aim of this work focuses on studying the response of GH/IGF-1 axis to glucagon administration and insulin-induced hypoglycemia and identifying the impact of this response on IGF-1 bioactivity, measured by cell-based KIRA assay.

2.1. Glucagon administration study

Among various medical procedures known to validate growth hormone (GH) deficiency in humans, the glucagon stimulation test (GST) is used (102-105). However, the mechanisms underlying glucagon-induced GH release are not fully understood.

GH regulates insulin-like growth factor 1 (IGF-1) secretion and circulating IGF-1 loop acts as a feedback mechanism at the pituitary gland level to increase GH (103). To date, the interplay between GH, IGF-1 and IGF-binding proteins determines IGF-1 bioactivity, the ability of IGF-1 to induce its signaling. Thus, IGF-1 bioactivity is sufficient to reflect any variety in GH/IGF-1 system (106;107).

The impact of insulin on GH/IGF-1 axis has been well studied. Insulin-induced stimulation of GH receptor expression, -down-regulation of IGFBP-1, -up-regulation of IGFBP-2 and -decrease in IGF-1 bioactivity have been reported (108-111). Nevertheless, there is no information about the interference of glucagon, as a counter-regulatory hormone of insulin, with GH/IGF-1 axis. In the current study, the aim was to investigate whether glucagon modulates IGF1 bioactivity and to determine how this change accounts later for GH stimulation.

Although the positive effects of high protein diet, characterized with increased glucagon secretion on weight control have been suggested, the increased incidence of cardiovascular disease related to the consumption of this diet has been demonstrated

(112-115). However, the effect of glucagon on IGF-1 bioactivity regarding induced cardiovascular disease is missed.

2.2 Insulin-induced hypoglycemia study

The interplay between insulin and GH/IGF-1 system has been well studied. To date, insulin is known to induce GH receptor expression, to down-regulate IGFBP-1 and to up-regulate IGFBP-2 (108-111). To be able to study IGF-1 bioactivity, as an index of free IGF-1, we have to take in consideration the inhibitory and stimulatory effects of IGF-binding proteins (106;107). The previous data from our lab showed that insulin administration during hyperinsulinemic-euglycemic clamp decreases IGF-1 bioactivity through up-regulation of IGFBP-2 (108). Blood glucose level during this clamp is held constant by glucose infusion with a certain concentration. However, some in vivo and in vitro investigations revealed that glucose inhibits GH release, interferes with GH/IGF-1 system and controls GHR expression and GH-induced IGF-1 expression (116;117). Moreover, oral glucose tolerance test, a test used to validate the presence of acromegaly and excess GH release, depends on the principle that GH secretion counter-regulates hypoglycemia (118). On the other hand, insulin tolerance test (ITT) is a medical procedure performed to assess the good function of the hypothalamo-pituitary-adrenal axis. Insulin injection during this test is able to induce in addition to hypoglycemia, GH release and the mechanism is unclear. Therefore, the current study is aimed to analyze dynamics of changes in secretion of GH/IGF-1 system members during applying of insulin tolerance test in humans and to verify if insulin-induced IGF-1 bioactivity response is preserved in hypoglycemic condition. In addition diabetes mellitus and its complication including cardiovascular disease (CVD) are responsible for more than million deaths worldwide every year. Insulin-treated diabetic patients who experience frequent severe hypoglycemia, as a result of intensive glucose control, show increased rate of mortality and the causes are unclear (119-121). However, changes in GH/IGF-1 axis related to the development of cardiovascular disease and increased death have been suggested. Moreover, IGF-1 bioactivity, as a crosstalk between GH, IGF-1 and IGF-binding proteins (IGFBPs), is considered as a survival predictor in humans (122;123). The current study is aimed to investigate GH/IGF-1 system response to insulin induced

hypoglycemia and its impact on IGF-1 bioactivity as a possible link between hypoglycemia and increased mortality.

3. Materials and methods

3.1. Materials

3.1.1. Laboratory materials and equipment

Cell culture flasks (75 cm^2)	Sarstedt, Nümbrecht, Germany
Sterile plastic tubes (10ml and 50 ml)	
Centrifuge	Roth, Karlsruhe, Germany Eppendorf, Hamburg, Germany
Squeegee for cell-culture flasks	TPP, Trasadingen, Switzerland
Sterile Pasteur-pipettes	Sarstedt, Nümbrecht, Germany
Microscope	Leica Mikrosysteme, Wetzlar, Germany
Cell-counter, Neubauer	VWR, Darmstadt, Germany
Cell Culture 48-well Plates, CELLLSTAR 48-Well	Greiner, Frickenhausen, Germany
5% CO2 incubator	Heraeus Instruments, Düsseldorf
Multi-channel pipettes	Eppendorf, Hamburg, Germany
Thermometer	Braun, Kronberg, Germany
Plate-shaker	Heidolph, DSG 304/M4, Schwabach, Germany
Plate-washer	Anthos, Fluido, Biochrom, USA
Delfia reader, Victor 1420	PerkinElmer LifeSciences), California USA
Sterile flow-bench	Heraeus, Hera safe, Hanau, Germany
Syringe	B Braun, Melsungen, Germany
Cryotubes	Eppendorf, Hamburg, Germany
Sterile pipettes (10 ml)	Eppendorf, Hamburg, Germany
Plastic boxes	B Braun, Melsungen, Germany
DELFIA Clear 96-well Stripplate,	(PerkinElmer LifeSciences), California, USA
Water bath	Medingen, SWB20, Arnsdorf, Germany
Analytical balance	Mettler Toledo, Gießen, Germany
PH-meter	VWR International LLC, USA
NanoDrop ND-1000	PeqLab, Erlangen, Germany

384-Well Mutliply® -PCR Plate	Sarstedt, Nümbrecht, Germany

3.1.2. Chemicals, reagents and buffers

Dimethyl-sulfoxide (DMSO)	Sigma, Munic, Germany
Dulbeccos Modified Eagle´s Medium, DMEM (with L-glutamine and 3,7g/l NaHCO3 and 1g/l glucose)	Biochrom, Berlin, Germany
Geneticin (G 418),	Biochrom, Berlin, Germany
Hygromycin (20 ml, 50 mg/ml	Gibco, Life Technologies, Darmstadt, Gemany
Penicillin/Streptomycin (100 ml,– 10000 ug/ml	Biochrom, Berlin, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
Human serum albumin (HAS)	MP BioMedicals, Eschwege, Germany
Human serum albumin	
Hepes 50mM	Sigma, Munic, Germany
NaCl	Merck, Darmstadt, Germany
$Na_4P_2O_2$,10H_2O	ICN-Biomedicals
NaF Merck, Darmstadt, Germany	
KCL Merck, Darmstadt, Germany	
$NaHCO_3$	Merck, Darmstadt, Germany
KH_2PO_3	Merck, Darmstadt, Germany
$Mg_2SO_4.7H_2O$	Merck, Darmstadt, Germany
99% Glycerol	Sigma, Munic, Germany
NP-40 (IGEPAL-ca 630	Bie og Berntsen
$MgCL_2.6H_2O$	Merck, Darmstadt, Germany
$CaCl_2.2H_2O$	Merck, Darmstadt, Germany
Tween	Merck, Darmstadt, Germany
Complete, EDTA-free Protease Inhibitor Cocktail	Roche scientific, Basel, Switzerland
Bovine serum albumin (BSA)	Sigma, Munic, Germany
Substrate reagent pack	R&D system, Pennsylvania, USA
Stop Solution 2N Sulfuric Acid	R&D system, Pennsylvania, USA

IGF- I, WHO,02/254 (10µg),	NIBSC, London, UK
Sodium orthovanadate	Sigma, Munic, Germany
PBS buffer (10x)	Merck, Darmstadt, Germany
Power SYBR green PCR master mix	Fisher scientific Applied Biosystems, schwerte, Germany
Proteases and phosphatases inhibitors	Roche scientific, Basel, Switzerland
Acetic acid	
TrisHCL	Roth, Karlsruhe, Germany
Sodium deoxycholate	Sigma, Munic, Germany
EDTA	Roth, Karlsruhe, Germany
SDS	Roth, Karlsruhe, Germany
Glycerol	Roth, Karlsruhe, Germany
β-Mercaptoethanol	Roth, Karlsruhe, Germany]
Bromphenolblue	Sigma, Seelze, Germany
Glycine	Roth, Karlsruhe, Germany
Methanol	Roth, Karlsruhe, Germany

3.1.3. Buffers

3.1.3.1 Cell culture solutions

10% FCS-DMEM

500 ml DMEM

50 ml FCS

2.5 ml penicillin/ streptomycin (1mg/ml)

2.5 ml hygromycin

250µl Geneticin

Storage at 4 C^0

3.1.3.2 Kinase receptor activation assay solutions

1M, $MgCl_2$,6H2O+$CaCl_2$,2H2O (10 ml)

2.033 g $MgCl_2$, 6H2O
1.4702 g $CaCl_2$, 2H2O
Sterile water up to 10 ml
Storage at -30 C^0

FT3-bicarbonatbuffer

12.94 g $NaHCO_3$
Sterile water up to 1000 ml

FT3-dialysevæske

34.7 g NaCL
1.77 g KCL
0.94 g $CaCl_2$, $2H_2O$
0.81 g KH_2PO_3
0.7 g $Mg_2SO_4.7H_2O$
Sterile water up to 1000 ml

Krebs Ringer buffer

16 ml FT3 bicarbonatebuffer
20 ml FT3 dialysevaeske
Sterile water to the final volume of 100 ml

Washing buffer

100 ml PBS buffer (10x)
900 ml sterile water
0.5 % Tween 20
Storage at 4 C^0

Lysis buffer (1 Liter)

50 mM Hepes
137 mM NaCl
10 mM $Na_4P_2O_7$,10 H_2O
10mM NaF
100 µl (<u>1M, $MgCl_2$,$6H_2O$+$CaCl_2$,$2H_2O$)</u>

900 µl Sterile water
PH adjusted to 7.4
10 ml NP-40
90 ml glycerol 99%
Storage at 4 C^0

Activated orthovanedate pH 10.0

200 mM solution of sodium orthovanadate is adjusted to pH 10.0 using either 1N NaOH or 1N HCL. After that it is boiled until its color disappears (approximately 10 minutes) and then it is cooled to room temperature. These steps are repeated until the solution becomes without any color and the pH stabilizes at 10.0.
Storage as aliquots at – 20C ^{0}

IGF-1 stock solutions

10 µg dissolved in 1ml acetic acid (0.2 M) containing 0.2 % HSA= (10 µg/ml)
Stock solutions of 400 µl with 1.25 mg/L are prepared and distributed in tubes of 40 µl
Storage at – 30C ^{0}

Detection antibody buffer:

500 ml PBS buffer (1X)
0.2% BSA (1g in 500 ml 1x PBS)
0.5% Tween 20
Storage at 4 C^0

3.1.3.3 Western blot solutions

RIPA Puffer

10 mM TrisHCl pH 7.2
150 mM NaCl
5 mM EDTA
1% Triton X-100
1% sodium deoxycholate
0.1% sodium dodecyl sulfate (SDS)

5x Laemmli Buffer

1 M Tris-HCl, pH 6,8, 6,26 % (v/v)

SDS, 2,3 % (w/v)
Glycerol 10 % (v/v)
-Mercaptoethanol, 5 % (v/v)
Bromphenolblue, 0,1 % (w/v)

10% polyacrylamide gel

4152µl H2O
2125µl 1,5M TrisHCl pH 8.8
2138µl Acrylamid 40
85µl 10% SDS
42.5µl 10% APS
8.5 µl TEMED

4% Stacking gel

1408µl H2O
550µl 0,5M TrisHCl pH 6.8
220µl Acrylamid 40%
22µl 10% SDS
11µl 10% APS
2.2µl TEMED

10 % SDS-PAGE-Running buffer

Tris-HCl, 15 g/l
Glycine, 72 g/l
SDS, 5 g/l
pH 8,3

10 % Transfer buffer

Glycine, 144 g/l
Tris, 30 g/l
SDS, 100 g/l
pH 8,3
Methanol 20%

Blocking buffer

Non-fat dried milk, 5 g
1 % PBS, 1 l

Washing buffer

Non-fat dried milk, 5 g
1 % PBS, 1 l
Tween 20, 1 ml

PBST

Tween 20, 100 µl
1 % PBS, 100 ml

Restore™ Western Blot Stripping Buffer	VWR International GmbH, Darmstadt, Germany

3.1.4. Kits

GlucaGen	Novo Nordisk Pharma Inc, Mainz, Germany
Glucometer Biosen 5130, EKF-diagnostic	Magdeburg, Germany
Insulin Elisa Kit	Mercodia, Uppsala, Sweden
Glucagon RIA kit	DPC Biermann, Bad Nauheim, Germany
Automated chemiluminescent immunometric assay	Immunodiagnostic systems GmbH, Frankfurt, Germany
IGFBP-1 ELISA kit	DSL Deutschland GmbH, Sinsheim, Germany
IGFBP-2 ELISA kit	DSL Deutschland GmbH
Human phospho-IGF1 receptor capture antibody Kit	R&D system
Rneasy Mini Kit	Qiagen, Hilden, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
cDNA Synthese Kit mit Rnase inhibitor	Fisher scientific Applied Biosystems, Schwerte, Germany
PCA protein assay kit	VWR Intrenational GmbH Darmstadt, Germany

3.1.5. Primers (Invitrogen, Darmstadt, Germany)

Mouse HPRT	5′-CAGTCCCAGCGTCGTGATTA-3′ 5′-AGCAAGTCTTTCAGTCCTGTC-3
Mouse IGF-1	5′-TTCACACCTCTTCTACCTGGCG-3′ 5′-CGAACTGAAGAGCATCCACCA-3′
Mouse IGFBP-1	5′-CATTCTTGTTGCA-3′ 5′-ACCTCAAGAAA-3′
Mouse IGFBP-2	5′-TCTCCTGCTGCTGC-3′ 5′-GGCGCGGGTACCTGTGAAAA-3′
Mouse IGFBP-3	5′-AATGGCCGGCGGGTTCTGC-3′ 5′-TTCTGGGTGTCTGTGTTT-3′

3.1.6. Antibodies

Monoclonal anti Phospho-Akt (Ser^{473})	Cell Signalling Technology Frankfurt, Germany
Monoclonal anti Akt	Cell Signaling Technology Frankfurt, Germany

3.1.7. Cell lines

Human embryonic kidney (HEK) cells that expressed the human IGF-1R was provided by Prof. J. Frystyk from institute of Clinical Medicine, Aarhus University, Denmark.

Mouse primary hepatocytes that were isolated by Stefanie Lieske from institute of nutritional science in Potsdam University

3.2. Methods

3.2.1 In vivo

3.2.1.1 Study protocol

The Ethical Committee of the Charité University Medicine Berlin was used to approve this study, whereas it is performed according to the Declaration of Helsinki. A written informed consent was obtained from each study participant. The trial was registered at ClinicalTrials.gov (registration number: NCT00929812).

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3.2.1.2 Study participants

In total, 37 subjects participated in this study, 11 obese healthy participants (OP), 13 lean healthy participants (LP) and 13 patients with type 1 diabetes mellitus **(Table 1)**. A physical examination, a set of hormonal tests and body composition were obtained. According to a full medical history, participants with type 2 diabetes mellitus, cardiovascular disease, an evidence of impaired hepatic and renal function, uncontrolled hypertension, any current inflammatory or malignant disease, pregnancy and treatment with any medication known to interact with hypothalamo-pituitary function or glucose homeostasis had been excluded, whereas an intact hypothalamo-pituitary function, determined by measuring fasting baseline levels of ACTH, cortisol, TSH, free T4, prolactin, LH, FSH, T, estradiol, and SHBG and performing insulin tolerance test and/or GHRH-arginine test for the assessment of GH and cortisol levels, had been considered as a critical inclusion. A 75 g-oral glucose tolerance test was performed to exclude the presence of type 2 diabetes mellitus or impaired glucose tolerance test in both lean and obese subjects.

3.2.1.3 Study design

All subjects received 1 mg glucagon (GlucaGen; Novo Nordisk Pharma Inc) intramuscularly for the glucagon study and both lean and obese participants performed insulin tolerance

tests for the insulin-induced hypoglycemia study. Serum samples were obtained at different time points and were kept frozen by -80 C0 until analyzed. Type 1 diabetic patients received the last dose of long-acting insulin in the evening before glucagon injection.

	Obese Subjects	**Lean Subjects**	**T1DM**
Age, years	28.4 ± 2.6	25.1 ± 0.6	32.3 ± 2.1[a]
Gender, males/females	5/6	6/7	6/7
Anthropometry			
Body mass index, kg/m^2	34.4 ± 1.7[a]	21.7 ± 0.6	24.8 ± 0.95[a]
Waist circumference, cm	108.9 ± 3.7[a]	79.6 ± 1.8	91.2 ± 3.6[a]
Waist to hip ratio	0.94 ± 0.02[a]	0.86 ± 0.01	0.9 ± 0.02[a]
Body composition			
Truncal fat mass, %	32.9 ± 2.3[a]	20.4 ± 1.6	24.2 ± 2
Truncal lean mass, %	39.3 ± 1.6	44.2 ± 1.4	44.7 ± 1.8
Biochemical parameters			
Fasting glucose, mmol/L	4.7 ± 0.3	4.5 ± 0.2	5.3 ± 0.4
Fasting insulin, pmol/L	62.1 ± 23.4[a]	32.9 ± 4.1	7.1 ± 0.1[a]
Fasting NEFAs, mmol/L	0.36 ± 0.05	0.37 ± 0.04	0.35 ± 0.07
Fasting glucagon, ng/L	42.7 ± 3.4	38.4 ± 3.95	41.7 ± 3.4
HOMA-IR	1.9 ± 0.24[a]	0.99 ± 0.13	

Table 1. **Baseline Characteristics of Obese, Type 1 Diabetic Patients and Lean Participants**

Values are presented as means ± SEM.

[a]P < 0.01 compared with LP.

3.2.1.4 Hormone assays

To estimate capillary blood glucose, glucose oxidase method was used. This method depends on the conversion of blood glucose to glucono -1 ,5 – lactone and hydrogen peroxide. A peroxidase enzyme that breaks down hydrogen peroxide to water and oxygen

is used forming with an oxygen acceptor like ortho toluidine coloured compounds that can be measured by colorimetric assays.

Serum insulin concentration was assessed by normal ElISA kit with inter- and intraassay coefficients of variation (CV) 3.6% and 4%, respectively.

Plasma glucagon concentration was measured by radioimmunoassay (RIA) using a constant concentration of radioactive I^{125}-labeled glucagon as a tracer that compete with unlabeled plasma glucagon on the limited binding sites of glucagon antibody raised in rabbit. Setting up a standard curve using certain dilutions of unlabeled glucagon enables to determine glucagon level in unknown samples with intra- and interassay CV were 4.8%and 8.6% respectively.

According to the manufacturer's protocol of commercially available automated chemiluminescent immunometric assay, serum IGF-1 was measured in one run by its acidic separation from its binding proteins, neutralizing and adding of excess IGF-2 that occupies binding sits of IGFBPs and prevents their binding to IGF-1. Free IGF-1 then was assessed using streptavidin coated magnetic particle, acridinium labeled anti-IGF-1 monoclonal antibody and biotin conjugated anti-IGF-1 monoclonal antibody. Using the same above mentioned chemiluminescent immunometric principle, serum HGH and IGFBP-3 levels were quantified in one run.

IGFBP-1 and IGFBP-2 were measured in one run and duplicate using ELISA kits.

Serum bioactive IGF-1 was measured using Kinase receptor activation assay described below.

3.2.2. In Vitro

3.2.2.1 Kinase Receptor Activation Assay (KIRA)

IGF-1 is found in human serum binding to different kinds of stimulatory and inhibitory IGFBPs. Free IGF-1 binds to IGF-1R leading to IGF1R phosphorylation on tyrosine residues. KIRA assay depends on stimulation of cells that have been transfected with human IGF-1R with serum and collecting both phosphorylated and un-phosphorylated IGF-1R using anti IGF-1R capture antibody. Anti-phospho-tyrosine antibody conjugated with horseradish peroxidase and a suitable substrate is used to detect the

phosphorylated IGF-1R induced by bioactive IGF-1.

3.2.2.1.1. Cell culture

3.2.2.1.1.1. Thawing of the cells

HEK cells that are stably transfected with the human IGF-1R were a gift from Prof. J. Frystyk, institute of Clinical Medicine, Aarhus University, Denmark. Cryotube with HEK cells was transferred from liquid nitrogen to a $37C^0$ hot water bath for 2 minutes. The outside of the cryotube was sterilized with 70% ethanol and the cells were transferred to 50ml-tube of 20 ml 10% FCS-DMEM and centrifuged at 850 g for 7 minutes to remove DMSO. Supernatant was discarded and 40 ml 10%FCS-DMEM were added and mixed using a sterile pipette. Cell suspension was transferred to and was incubated in 5% CO_2 incubator.

3.2.2.1.1.2 Freezing of the cells

In contrast to the thawing, freezing process should be slow to prevent crystal growth in the cells. The cells were removed carefully from the bottom of the culture flask and transferred to 50 ml-tube. They were centrifuged at 22 C^o and 800 g for 7-8 minutes and the supernatant was discarded. 8 ml of freezing solution (10% DMSO+ 90% FCS) was added to the cells and the re-suspended cells were distributed to 8 sterile cryotubes. The cells were kept at the beginning at -80 C^0, hereafter they were transferred to another container with liquid nitrogen.

3.2.2.1.1.3 Cultivation of the cells

After thawing, cells were grown in 40 ml 10% FCS-DMEM in 75 cm^2-cell culture flasks, in a humidified atmosphere with 5 % CO2 at 37 °C. HEK cells grow adherent to the ground of the flask. Every three days the medium was thrown away and the cells were supplemented with fresh 10% FCS-DMEM medium. When the cells reached 80% confluent, they were passaged by removing 30 ml of the medium and harvesting the cells

from the bottom of the flask in the remaining 10 ml medium using a scarper. Cells then were centrifuged at 22 C^0 and 800 g for 7-8 minutes, supernatant was removed again and 10 ml of 10% FCS-DMEM medium was added. Cells were counted and needed amount of cells were seeded in 75 cm^2-cell culture flasks.

3.2.2.1.2 Assay performance

Measuring IGF-1 bioactivity using KIRA assay takes 4 days

KIRA, day 1

The cell culture flask was taken from the incubator and the cells were controlled under the microscope. The cells were then removed gently with the Squeegee and transferred to a 50 ml tube and centrifuged at 22 C^0 and 800 g for 7-8 minutes. Supernatant was discarded and 10 ml of DMEM with 10% FCS was added. From this cell suspension, a dilution of 10 µl cell suspension and 990 µl DMEM medium or 100 µl cell suspension and 900 µl DMEM medium depending on the cell number expected, was made. Cells were counted. According to the cell density, a 800,000 cells /ml-cell suspension was made and 500 µl from this cell suspension was put in every well in a 48-well plate with mixing the cell suspension between every row or between each refilling of the syringe. The plate was incubated at 37 C^0, 5% CO2 for the next day and a new passage was started using 2-3 ml of the cell dilution (depending on the cell density) with 38ml serum-DMEM medium in a 75-cell culture flask and incubated at 37 C^0, 5% CO2 for the next week.

KIRA, day 2

All cell media were discarded from the 48-well plate and 500 µl serum-free medium with 0.1 % (human serum albumin) HSA was added to every well and incubated at 37 C^0, 5% CO2 for the next day. phospho IGF1R antibody was diluted (40µl in 10 ml 1x PBS) to get at the end a concentration of 5.76 µg/ml and the Delfia plate was coated with the diluted phospho IGF1 R antibody (100 µl/well) and incubated at 4 C^0 for the next day.

KIRA, day 3

All samples measured in KIRA assay were human serum samples. While serum samples were thawed slowly on ice, blocking buffer was prepared using 18 ml 1xPBS + 2ml HSA (20%) and 200 µl of this blocking buffer was added to every well. The Delfia plate was then incubated at RT for at least 1 hour. KRB (krebs ring buffer) buffer was made, covered with a film and gased with 5% CO2 for 8 minutes. Finally, 200 µl HSA were added to 100 ml KRB to serve as a protein carrier for IGF-1 later. To 50 ml lysis buffer, 1 EDTA-free tablet+ 2 ml activated orthovanadate solution were added and put on the ice until used. To create IGF-1 standard curve, we had to generate serial dilutions of known concentrations IGF-1 in KRB buffer, including 0, 1, 2, 4, 8 µg/L and incubate them in a box with 37 C^0 water. After thawing of serum samples, they were diluted (1:100) in KRB buffer and incubated with IGF-1 standards in the same box of hot water. At the same time the 48-well plate with HEK cells were put in another box filled with 45 C^0 hot water for at least 15 minutes. The media was removed from the 48-well cell plate, two wells at the same time and 500 µl from standards or samples were added and again in two wells at the same time. For every two wells together it took 30 seconds for medium removing and sample filling.

After exactly 16 minutes the stimulation of the cells was stopped by removing the standards or samples and adding 200 µl above described lysis buffer to every well. This should be done also in duplicates and in 30 seconds for every two wells together. The 48-well cell plate with lysis buffer was then incubated for at least one hour on the plate shaker at 4 C^0. During this hour, 300 µl fetal calf serum (FCS) was added to the rest of lysis buffer and kept at 4 C^0 until used. The coated and blocked 96-well delfia plate was washed with the washing buffer. From each well of the 48-well cell plate, 75 µl cell lysate as well as 25 µl (Lysis buffer with FCS) were added to a well of the 96-well Delfia plate, this should be done also in duplicate to get at the end Quadruplicate. At the end the 96-well Delfia plate was incubated overnight on plate shaker at 4 C^0 in the cold room for the next day.

KIRA, day 4

The plate was washed with the washing buffer and 100 µl from 1:360 in 1x PBS diluted anti-phospho tyrosine-HRP capture antibody was added to every well and left on the plate shaker at room temperature for two hours. 5ml of both Reagent A and B from the

substrate reagent pack were mixed and 100µl of this mixture were added to each well and incubated 10 minutes at RT. The reaction was stopped using 50 µl/ well of the stop solution and the signal was read using Delfia reader, Victor 1420.

3.2.2.2 Western blot

Mouse primary hepatocytes were isolated kindly by Stephanie Lieske from Potsdam University and treated with glucagon (10 nM, 24hr) in the presence or absence of growth hormone (300 ng/ml, 24hr). After treatment, cells were lysed in a RIPA buffer with protease and phosphatase inhibitors. The protein concentration was measured by BCA Protein Assay. After incubation with 1x Laemmli buffer at 95 C^{o} for 10 minutes, 30 µg proteins from primary hepatocytes were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS/PAGE for 3-4 hours at 25 mA and blotted on a nitrocellulose membrane for detection procedure according to Laemmli protocol (124). The membrane then was blocked with Tris-buffered saline-Tween (TBST) containing 5% non-fat dried milk, pH 7.4 for 1 hour at room temperature and washed with TBS-T. Monoclonal antibodies against Phospho-Akt (Ser^{473}) and Akt (pan) were diluted 1: 1000 according to manufacturer's recommendation. The blotted membranes were incubated with the primary antibodies over night at 4 °C followed by washing with TBS-T and incubation with 1: 5000 diluted secondary antibody for 1 hour at room temperature. Since membranes were used to detect more than one protein, membranes were stripped using Restore™ Western Blot Stripping. After developing the membranes with Lumi-Light Western Blotting Substrate the detection was done by Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software (BIO-RAD Laboratories GmbH, Munich, Germany).

3.2.2.3. Gene expression

3.2.2.3.1 Isolation and treatment of mouse primary hepatocytes

Mouse primary hepatocytes were isolated by Stephanie Lieske as described previously (125) and then incubated with glucagon (10 nM, 5hr) in the presence or absence of growth hormone (300 ng/ml, 24hr). In a subsequent experiment different concentrations of glucagon (0.1, 1, 10, 100 nM) were used to evaluate glucagon-mediated dose-dependent effect on the transcription of IGF-1 system members. All stimulations were performed in four independent experiments done in duplicate. Glucagon-mediated dose-dependent effect experiment was performed in three independent experiments done in three technical repeats.

3.2.2.3.2 RNA extraction

Total RNA was extracted from stimulated mouse primary hepatocytes using the manufacturer instructions of the RNeasy Mini Kit. Cells were lysed and homogenized using a guanidine-thiocyanate-containing buffer, which ensures inactivation of RNases. The binding of intact RNAs to a silica gel membrane of spin columns was performed after adding of ethanol, whereas the contaminants were removed by different wash steps. Finally, RNAs were eluted in water, collected and quantified by measuring the absorbance at 260 nm using NanoDrop® ND-1000 Spectrophotometer. The ratio of the absorbance at 260 nm and 230 nm was used to qualify the RNAs and to determine the contamination with proteins or ethanol. The RNA samples were stored at -80C^{o} until cDNA synthesis.

3.2.2.3.3 cDNA synthesis

cDNA synthesis was done following the instructions of the commercial High Capacity cDNA Reverse Transcription Kit. RT Master Mix was prepared as described in **(Table 2)**

Substance	Volume (µl)
10x RT Buffer	2,0
25x dNTP Mix	0,8
10x RT Random Primers	2,0
Reverse Transcriptase	1,0
RNase inhibitor	1,0
RNases and DNases free water	2,3
Total per reaction	10,0

Table 2. Preparation of RT Master Mix for one reaction

1000 ng of the extracted mRNAs were mixed with RT Master Mix and reverse transcribed in a thermal cycler. Firstly the random primers annealed to the mRNA strand at 25° C for 10min. This step enables the binding of reverse transcriptase and synthesis of a complement DNA strand to mRNA at 37° C for 120min. the transcription is terminated at 85° C for 5min and hold at 4° C. Synthesized cDNAs were kept at-20° C until use.

3.2.2.3.4 Real-time reverse transcriptase polymerase chain reaction (RT–PCR)

qRT-PCR is a method used worldwide to study the expression of a target gene by amplification and quantification of a created cDNA of this gene using the following 10X Power SYBR Green Master Mix.

Substance	Volume (µl)
2x MM Buffer	2.5
Primer Forward	0.35
Primer Reverse	0.35
RNase and DNase-free water	0.8
Total volume	4

Table 3. Preparation of 10X Power SYBR Green Master Mix for one reaction

In every PCR cycle double DNA strand is denatured and separated into two strands at 95° C followed by annealing and binding of the primer at 60° C and polymerase-dependent elongation of every single DNA strand at 70° C. SYBR green dye is known to bind the double strand DNA emitting fluorescence signal. The cycle with lower fluorescence signal detected is called threshold cycle Ct and refers to the cycle number at which the exponential phase of amplification starts, whereas the specificity of this process is measured by determination of melting curve. The qualification was performed by using standard dilution series obtained from samples pools and generation of a standard curve with X-axis representing the quantity and Y-axis indicating Ct values. The analysis was performed using ViiA7 RUO software for real time PCR system version 1.2 and the obtained expression was normalized depending on the expression of mouse hypoxanthine phosphoribosyltransferase 1 (HPRT1) gene as a house keeping gene.

3.2.3 Statistical analyses

Statistical analyses for human study were performed using SPSS 19 (SPSS, Chicago, IL, USA). All data are expressed as means ± SEM. Baseline characteristics were compared as previously described (126).

Serial changes in glucose, insulin, glucagon, GH, total IGF-1, IGFBP-3, IGFBP-1, IGFBP-2 and IGF-1 bioactivity were analyzed using ANOVA for repeated measures. When the ANOVA was significant, changes were compared to baseline values using Student t-test for paired analysis in case of normally distributed data. In case of skewed data, the non-parametric Wilcoxon test was used. All significances were two-sided and p-values were regarded as statistically significant after correction by Bonferroni for multiple testing. The baseline value was calculated as the mean of the -30 and 0 min values. AUCs, calculated by the trapezoid method, were used to compare the time-courses of each parameter.

Data from animal and cell culture studies were derived from at least three independent experiments, performed in triplicates or in duplicate. Differences between groups were analyzed for significance using student t-test. Statistical significance was assumed when $p < 0.05$.

4. Results

4.1 Glucagon administration study

4.1.1 In vivo study

Plasma glucagon increased significantly after intramuscular glucagon administration by approximately 8-to 9-fold in all study groups with a peak detected after 30 minutes (324.8 ± 30.2 ng/L [OP], 355.9 ± 21.7 ng/L [LP] and 340.6 ± 25.6 ng/L [T1DM]) and returned to baseline levels by 240 minutes. The glucagon AUC240 increased in all study groups (800 ± 71.9 [LP], 921.4 ± 96.4 [T1DM], and 912± 93[OP]; P<.01) and was comparable between groups (P=0.3-0.4) (126).

In type 1 diabetic patients glucose levels increased maximally after 60 min with slight decrease towards baseline after 240 min., whereas only slight increase followed by decrease towards baseline levels was observed in LP and OP as previously described (126).

Plasma insulin concentrations did not change after glucagon or placebo administration in patients with type 1 diabetes (p=0.2-0.6), whereas plasma insulin levels showed an increase with a peak after 30 min followed by a decrease towards baseline level after 120 min. in LP and OP (126).

Glucagon induced a significant reduction in serum IGF-1 bioactivity in all study groups **(Table 4) (Fig. 3 A, B, C)**, whereas no changes in total IGF-1 and IGFBP-3 were detected **(Fig. 4)**. The glucagon-induced reduction in IGF-1 bioactivity was associated with a significant increase in GH levels in all study groups **(Table 4) (Fig. 3 D, E, F)**.

	Baseline	**60 min.**	**120 min.**	**180 min.**	**240 min.**
IGF-1 bioactivity (µg/l)					
LP	2.1±0.2	1.51±0.18*	1.34±0.13*	1.16±0.15*	1.27±0.15*
T1DM	1.9±0.3	1.29±0.18*	1.05±0.18*	1.4±0.2*	1.59±0.26
OP	2.1±0.3	1.39±0.15*	1.24±0.2*	1.37±0.15*	1.62±0.19
IGFBP-1 (ng/ml)					
LP	43.4±6.4	92.2±7.6*	79.2±5.1*	36.8±4	30.8±5.3*
T1DM	57.4±9.9	103.2±12.7*	112.8±13.6*	63.3±11.3	32.6±7.3*
OP	10.4±2.4	34.2±8*	36.7±6.2*	11.2±2.5	8.2±1.8
IGFBP-2 (ng/ml)					
LP	547±75	736±82*	758±89*	729±76*	667±87*
T1DM	435±84	628±94*	587±87*	621±98*	552±68*
OP	223±44	298±56*	278±45*	271±40*	266±36
	Baseline	**60 min.**	**150 min.**	**180 min.**	**240 min.**
GH (µg/l)					
LP	1.04±0.4	0.66±0.2	15.6±3*	10.7±2.1*	4.1±1.5
T1DM	1.2±0.3	3.4±0.97	16.6±3.1*	9.7±2*	1.9±0.4

OP	1.1±0.5	0.98±0.8	6.5±1.5*	3.9±1.2*	0.7±0.2

Table 4. Time courses of IGF-1 bioactivity, GH, IGFBP-1 and IGFBP-2 in lean participants, type 1 diabetic patients and obese participants after intramuscular glucagon administration.

Values are presented as means ± SEM. *p<0.01 as compared to baseline levels.

In all study groups, serum IGFBP-1 levels increased maximally after 60-120 min. followed by decrease towards baseline levels after 240 min. **(Table 4) (Fig. 5 A, B, C)**, whereas a sustained increase in IGFBP-2 levels was observed **(Table 4) (Fig. 5 D, E, F)**.

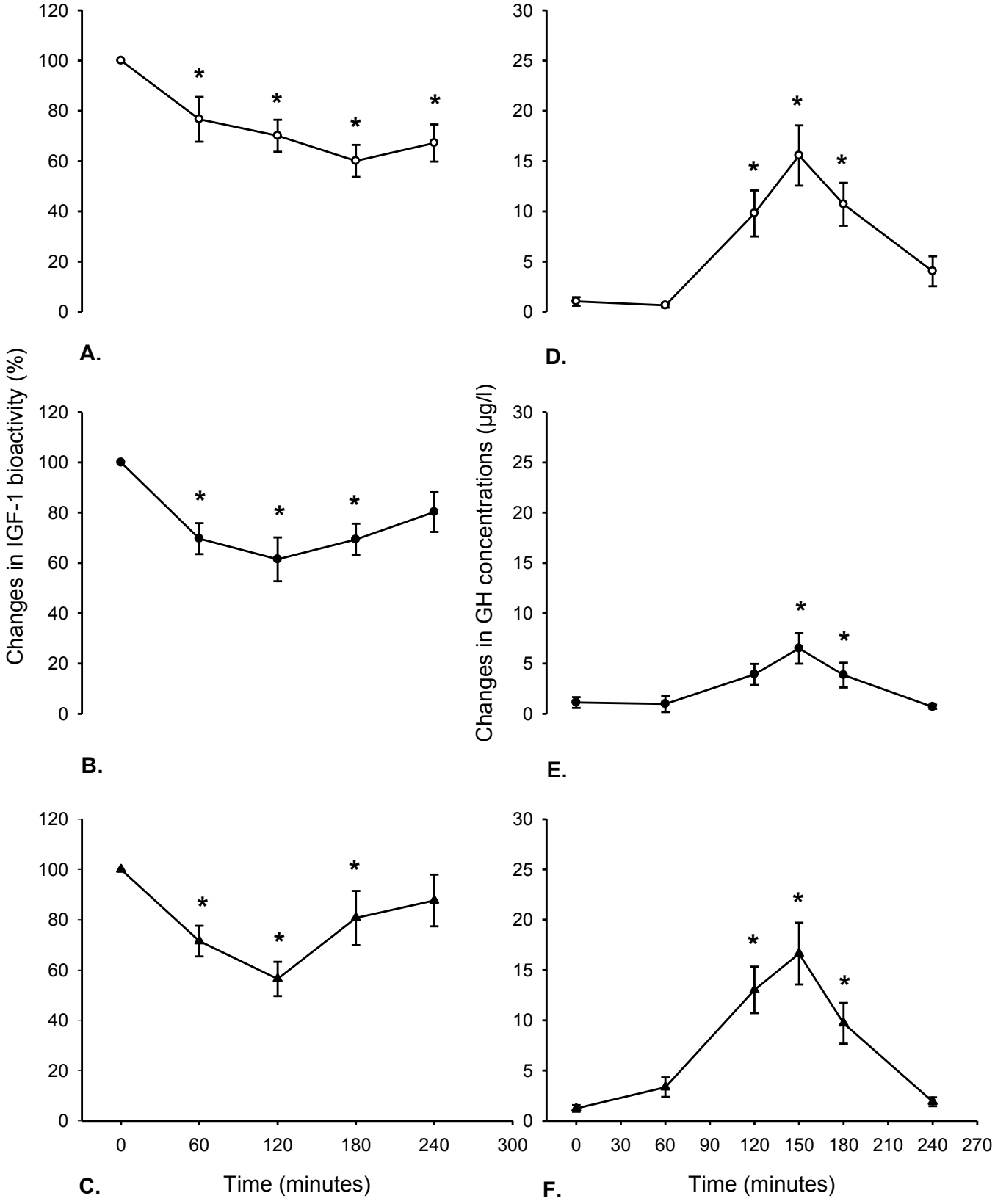

Changes in IGF-1 bioactivity (%)
Changes in GH concentrations (µg/l)
A.
B.
C.
D.
E.
F.
Time (minutes)
Time (minutes)

Figure 3. Mean (±SEM) changes in IGF-1 bioactivity after the administration of glucagon in **A.** 13 lean participants, **B.** 11 obese participants and **C.** 13 patients with type 1 diabetes mellitus. (* $p < 0.01$). Mean (±SEM) changes in growth hormone (GH) concentrations in **D.** 13 lean participants, **E.** 11 obese participants and **F.** 13 patients with type 1 diabetes mellitus. (* $p < 0.01$). The 0 min value represents the mean of two baseline values (-30 and 0 min). All values are calculated relative to the baseline.

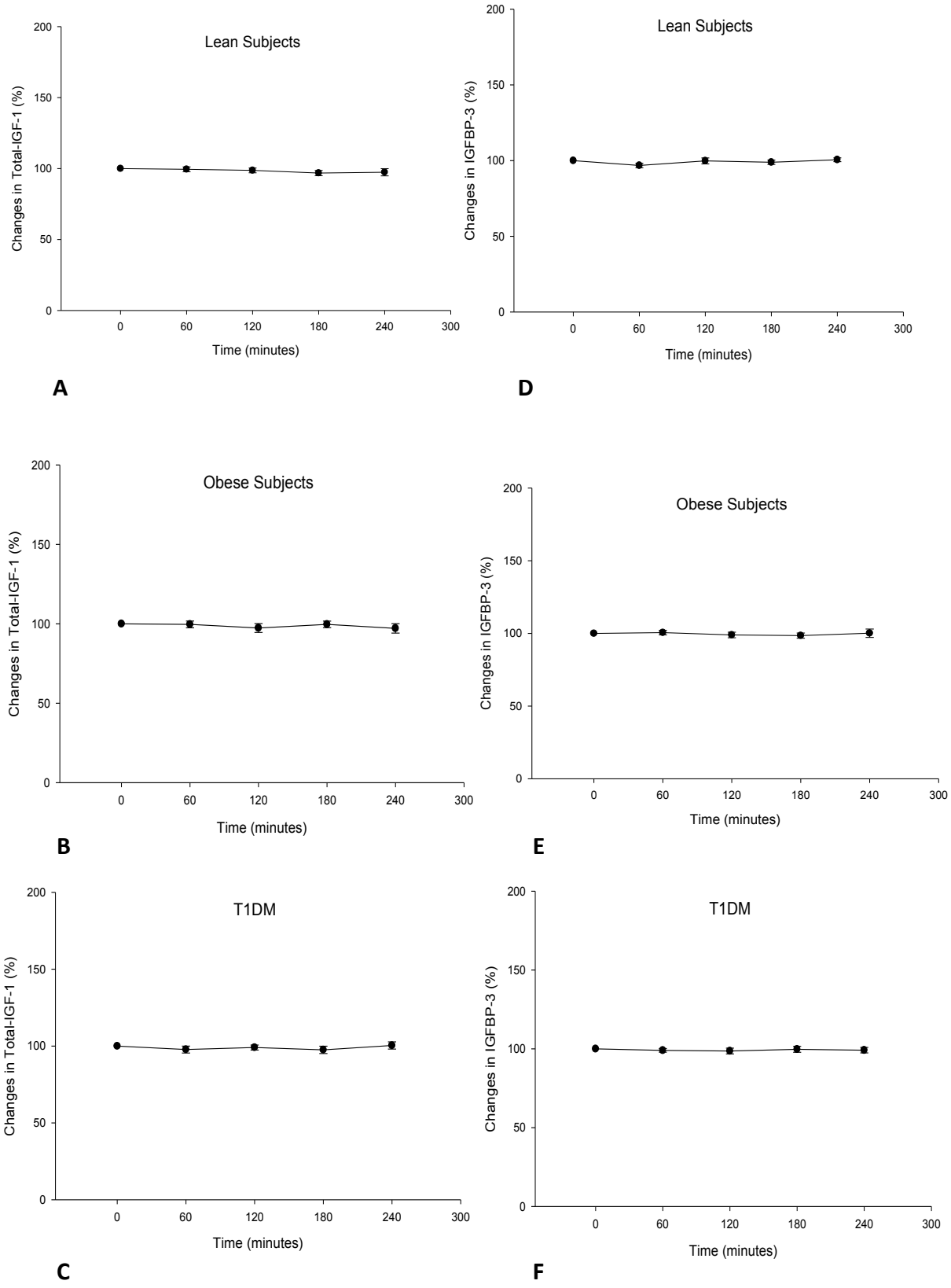


Figure 4. No changes in total IGF-1 **(A, B, C)** or IGFBP-3 **(D, E, F)** concentration were observed after glucagon administration in lean, obese participants and patients with type 1 diabetes mellitus.

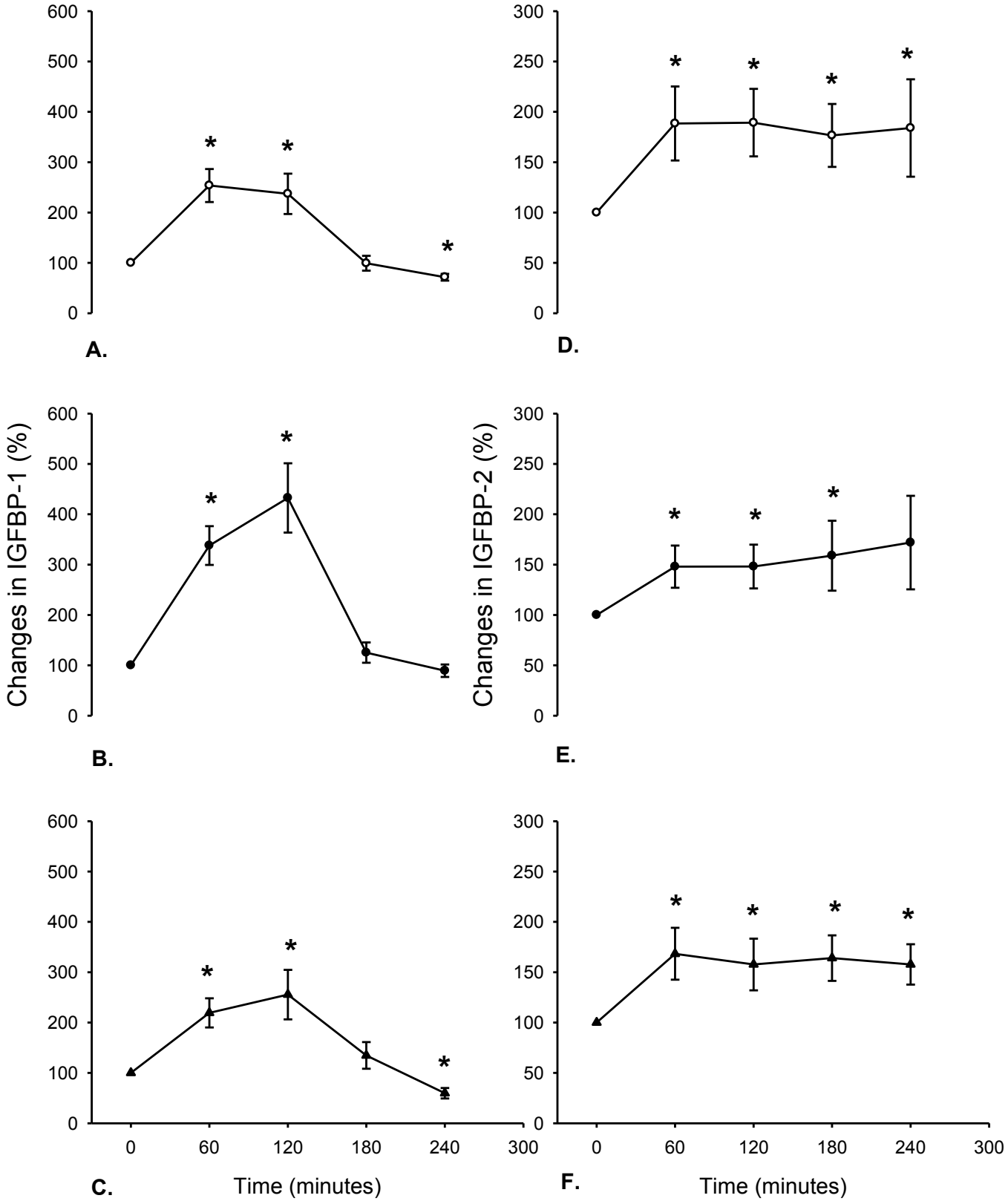

Changes in IGFBP-1 (%)
Changes in IGFBP-2 (%)
Time (minutes)
A.
B.
C.
D.
E.
F.

Figure 5. Mean (± SEM) changes in IGF-binding protein-1 (IGFBP-1) after the administration of glucagon in **A.** 13 lean participants, **B.** 11 obese participants and **C.** 13 patients with type 1 diabetes mellitus. (* $p < 0.01$). Mean (±SEM) changes in IGF-binding protein-2 (IGFBP-2) concentrations in **D.** 13 lean participants, **E.** 11 obese participants and **F.** 13 patients with type 1 diabetes mellitus. (* $p < 0.01$). The 0 min value represents the mean of two baseline values (-30 and 0 min). All values are calculated relative to the baseline.

4.1.2 In vitro study

4.1.2.1 Impact of glucagon, IGFBP-1, IGFBP-2 and IGFBP-3 on auto-phosphorylation of IGF1R

In order to determine the IGF-1-dependent effects of IGFBPs (IGFBP-1, IGFBP-2 and IGFBP-3) as well as the direct impact of glucagon on auto-phosphorylation of IGF1R, human embryonic kidney cells (HEK) were plated in 48-well plate and the KIRA assay was performed as described above by stimulating the cells with glucagon (10 µM, 100 µM), IGF-1 (2ng/ml) or IGF1LR3 (2ng/ml), an IGF-1 analogue that binds IGF1R and induces the same IGF-1 signaling without IGFBPs binding, with or without various concentrations of IGFBP-1, IGFBP-2 and IGFBP-3. Neither 10 µM nor 100 µM of glucagon induced direct auto-phosphorylation of IGF1R **(Fig. 6)**. IGFBP-1 at 0.1, 0.2, 1 and 2 nM reduced IGF-1 bioactivity by 85 ± 7, 72 ± 2, 45 ± 7, 23 ± 9 % of IGF-1-treated without IGFBP control respectively **(Fig. 7 A)**, whereas, the presence of IGFBP-1 did not affect IGF1LR3-induced phosphorylation of IGF1R **(Fig. 7 B)**. At concentrations of 0.4 nM and 1 nM, IGFBP-3 reduced IGF-1 bioactivity significantly by 72 ± 5 and 46 ± 5% respectively **(Fig. 7 E)**. However, IGFBP-3 did not show any inhibitory influence on IGF1LR3 signaling **(Fig. 7 F)**. The inhibitory effect of IGFBP-2 (63 ± 9 and 48 ± 6%) was observed by using concentrations of 100 and 200 nM respectively **(Fig. 7 C)** and was diminished by using IGF1LR3 **(Fig. 7 D)**, suggesting a much less IGF-1-dependent potency compared with IGFBP-1 and IGFBP-3.

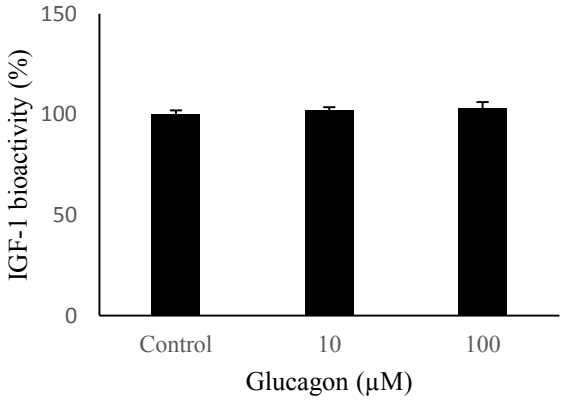
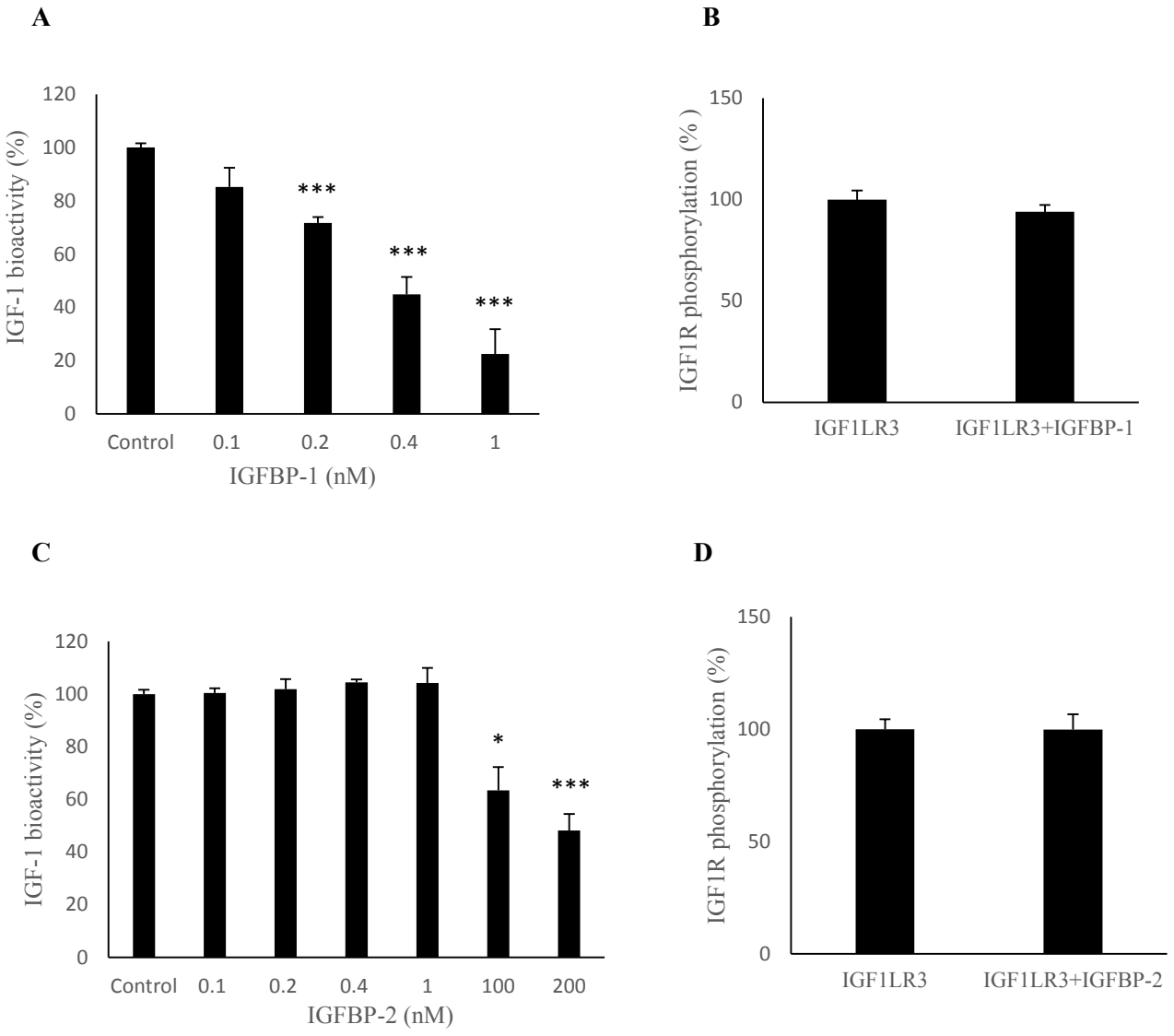


Figure 6. No direct effect observed of glucagon on IGF-1 bioactivity, determined by KIRA method. Values are presented as a percentage of control treated with IGF-1 (2 ng/ml) of four independent experiments done in duplicates.



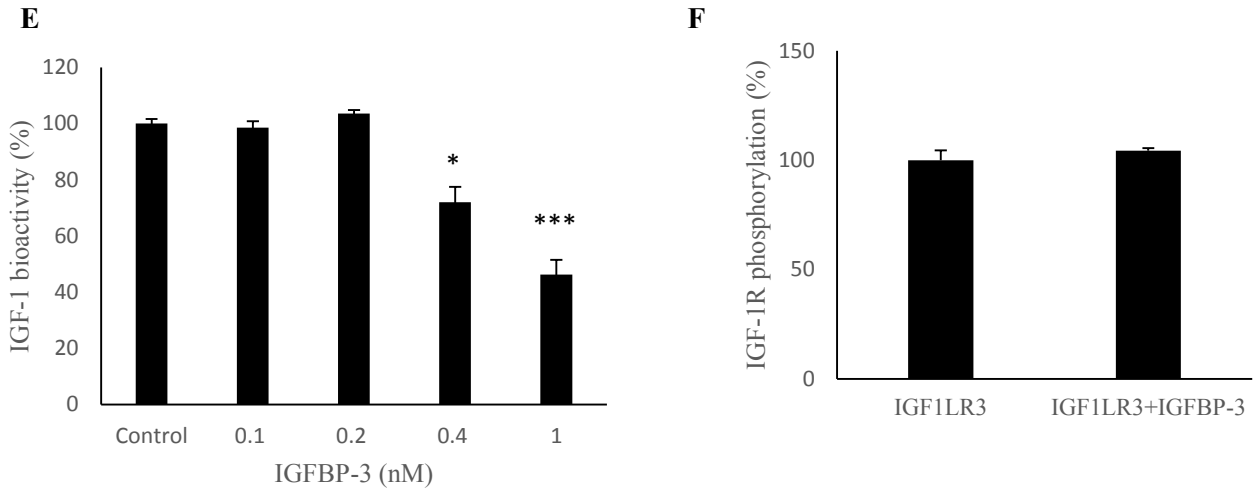
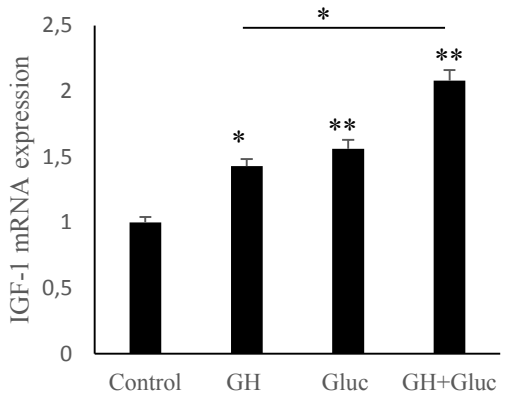


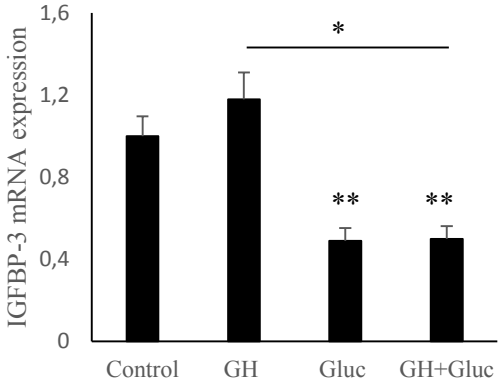
Figure 7. Dose-dependent effect of IGFBP-1, IGFBP-2 and IGFBP-3, respectively on IGF-1 bioactivity **A, C, E**. Effects of IGFBP-1(1nM) **(B)**, IGFBP-2(200nM) **(D)** and IGFBP-3(1nM) **(F)** on IGF1R3-induced phosphorylation of IGF1R, determined by KIRA method. Values are presented as a percentage of control treated with either IGF-1 (2 ng/ml) **(A, C, D**) or with IGF1LR3 (2 ng/ml) without IGFBPs **(B, D, F)**. Four replicates were performed for each treatment. * $P < 0.05$, *** $P< 0.001$.

4.1.2.2 Impact of glucagon on IGF-1 system gene expression and Akt phosphorylation in mouse primary hepatocytes

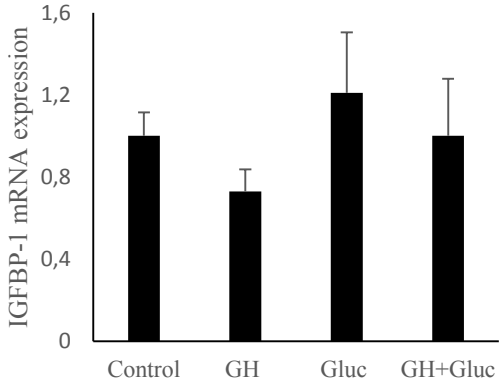
The effect of glucagon on gene expression of IGF-1, IGFBP-1, IGFBP-2 and IGFBP-3 was measured in freshly isolated mouse primary hepatocytes to see whether glucagon interferes with GH/IGF1-axis on the transcriptional level. Glucagon increased IGF-1 mRNA and decreased IGFBP-3 mRNA levels in mouse primary hepatocytes in a concentration-dependent manner **(Fig.8 E, F)**. This effect of glucagon was significant at concentration of 10 nM **(Fig.8 E, F)**. Treatment of mouse primary hepatocytes with 300 ng/ml GH alone for 24 hours significantly increased expression of IGF-1 mRNA to 143% ± 5% of the control levels, as expected and addition of 10 nM glucagon was able to increase basal and GH-stimulated IGF-1 mRNA to 156% ± 7% and 208% ± 8% of control levels respectively **(Fig. 8 A)**. GH tended to increase the transcription of IGFBP--3 to 118% ± 13%, although this increase was not significant **(Fig. 8 B)**. Glucagon (10 nM) decreased basal and GH-stimulated IGFBP-3 to 49% ± 6% and 50% ± 6% of control levels respectively **(Fig. 8 B)**. Glucagon tended also to produce higher IGFBP-1 and IGFBP-2 to 121% ± 29% and 111% ± 3%, although these effects were not statistically significant **(Fig. 8 C, D)**. Using western blot analysis, no phosphorylation of Akt after cell treatment with glucagon, GH or both of them was detected **(Fig. 8 G).**


IGF-1 mRNA expression
2,5
2
1,5
1
0,5
0
*
*
**
**
Control
GH
Gluc
GH+Gluc

A


IGFBP-3 mRNA expression
1,6
1,2
0,8
0,4
0
*
**
**
Control
GH
Gluc
GH+Gluc

B


IGFBP-1 mRNA expression
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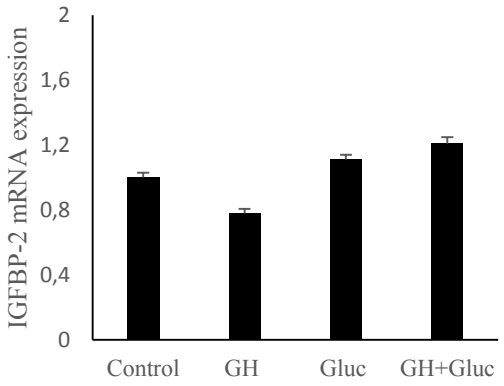
1,2
0,8
0,4
0
Control
GH
Gluc
GH+Gluc

C


IGFBP-2 mRNA expression
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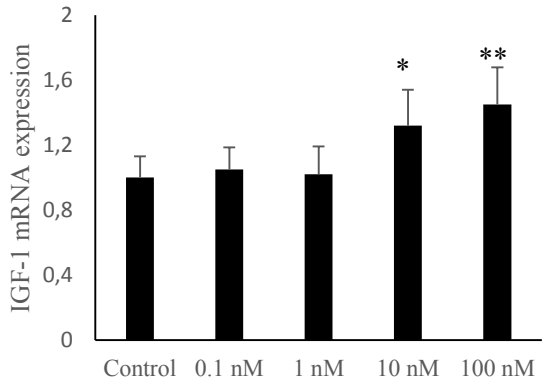
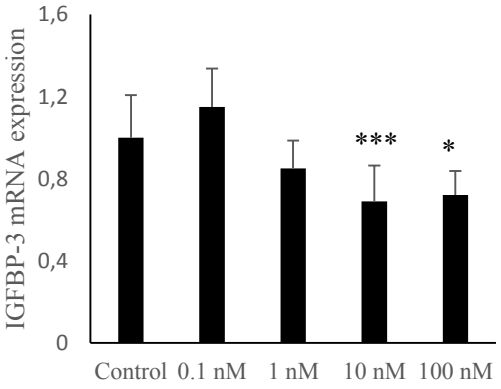
1,6
1,2
0,8
0,4
0
Control
GH
Gluc
GH+Gluc

D


IGF-1 mRNA expression
2
1,6
1,2
0,8
0,4
0
*
**
Control
0.1 nM
1 nM
10 nM
100 nM

E


IGFBP-3 mRNA expression
1,6
1,2
0,8
0,4
0
***
*
Control
0.1 nM
1 nM
10 nM
100 nM

F

G

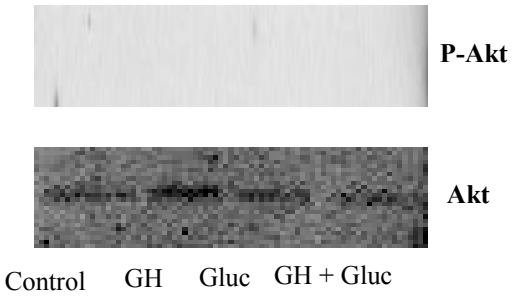


Figure 8. Relative changes of IGF-1 **(A)**, IGFBP-3 **(B)**, IGFBP-1 **(C)** and IGFBP-2 **(D)** mRNA expression in mouse primary hepatocytes after treatment with GH, glucagon or both of them. Dose-dependent effect of glucagon on IGF-1 and IGFBP-3 mRNA expression respectively (**E, F)**. Akt phosphorylation after stimulation of mouse primary hepatocytes with GH, glucagon or both of them **(G)**. Bars show means ± SEM of four independent experiments done in duplicate **(A, B, C, D)** or of three independent experiments done in triplicate **(E, F)**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$**.**

4.2 Insulin-induced hypoglycemia study

4.2.1 In vivo study

In both study groups, insulin administration induced an increase in plasma insulin with a peak detected after 15 minutes (509.4 ± 89.3 mU/L [LP] and 1017.1 ± 253.7 mU/L [OP]) and returned to baseline levels by 120 minutes **(Table 5) (Fig. 9 A, B)**. As expected glucose levels were decreased significantly after insulin injection in both groups by 15 and 30 minutes followed by increase towards baseline levels **(Table 5) (Fig. 9 C, D)**. Despite no changes in total IGF-1 and IGFBP-3 concentrations were observed **(Table 5) (Fig. 10 C, D, E, F)**, insulin induced a significant reduction in serum IGF-1 bioactivity **(Table 5) (Fig. 10 A, B)** and a significant increase in growth hormone levels in both study groups **(Table 5) (Fig. 10 G, H)**.

A significant decrease in serum IGFBP-1 was detected by 15 min. after insulin injection and followed by increase above the baseline levels by 120 min in both study groups **(Table 5) (Fig. 9 E F)**. A significant increase in IGFBP-2 that returned to the baseline levels by 120 min. was observed in both study groups **(Table 5) (Fig. 9 G, H)**

	Baseline	15 min.	30 min.	45 min.	60 min.	90 min.	120 min.
Glucose (mmol/L)							
LP	4.6 ± 0.14	2.4 ± 0.19*	2.3 ± 0.17*	3.4 ± 0.16*	4.1 ± 0.27	4.2 ± 0.12*	4.5 ± 0.07
OP	4.5 ± 0.11	2.8 ± 0.16*	2.1 ± 0.13*	3.2 ± 0.13*	3.9 ± 0.12*	4.3 ± 0.14	4.5 ± 0.15
Insulin (mU/L)							
LP	51.4 ± 6	509.4 ± 89.3*	222.7 ± 65.5*	75.7 ± 9*	82 ± 17.5	88 ± 16.5	85.3 ± 17.6
OP	81.7 ± 6.6	1017.1 ± 253.7*	283.2 ± 64.2*	110.3 ± 20.6	93.7 ± 15.5	73.8 ± 9.2	88.4 ± 20.7
IGFBP-1 (ng/ml)							
LP	43 ± 5.8	35.6 ± 5.1*	35.2 ± 5.4*	32.8 ± 5.2*	29.5 ± 4.6*	32.4 ± 5.4	57.3 ± 7.7
OP	16.5 ± 3.8	8.9 ± 3.5*	8.9 ± 3.2*	8.2 ± 2.8*	7.2 ± 2.3*	14.1 ± 3.6	35.5 ± 7.3*
IGFBP-2 (ng/ml)							
LP	647 ± 82.6	790 ± 93.9*	834.5 ± 106.5*	834.2 ± 95.7*	854.2 ± 99.2*	862.5 ± 104.7*	827 ± 94*
OP	324 ± 57	418 ± 67.9*	434.3 ± 69*	427.2 ± 67.1*	415.3 ± 62.8*	420.6 ± 67.4*	409.4 ± 60.9*
B-IGF-1 (µg/L)							
LP	2.28 ± 0.28		1.85 ± 0.18		1.76 ± 0.18*		1.69 ± 0.28*
OP	1.84 ± 0.15		1.69 ± 0.12		1.62 ±0.13*		1.44 ±0.14*

T-IGF-1 (ng/ml)							
LP	178.8 ± 9.7		183.5 ± 11.9		170.4 ± 12.6		175.6 ± 8.5
OP	185.2 ± 18.5		180.6 ± 14.8		177.9 ± 15.2		177.5 ± 16.1
IGFBP-3 (ng/ml)							
LP	4232.3 ± 149.1		4255.9 ± 139.7		4106.2± 90.3		4469.4 ± 307.8
OP	4410.4 ± 176.9		4504.3 ± 161.4		4389.6 ± 136.6		4375.4 ± 152
GH (µg/L)							
LP	3 ± 1		6 ± 1.5		14.4 ± 2.7*		3.9 ± 1.3
OP	0.9 ± 0.5		0.9 ± 0.4		9.3 ± 2.5*		2.1 ± 0.9

Table 5. Time courses of glucose, insulin, IGFBP-1, IGFBP-2, IGF-1 bioactivity, total IGF-1, IGFBP-3 and GH after applying of insulin tolerance test in obese and lean participants.

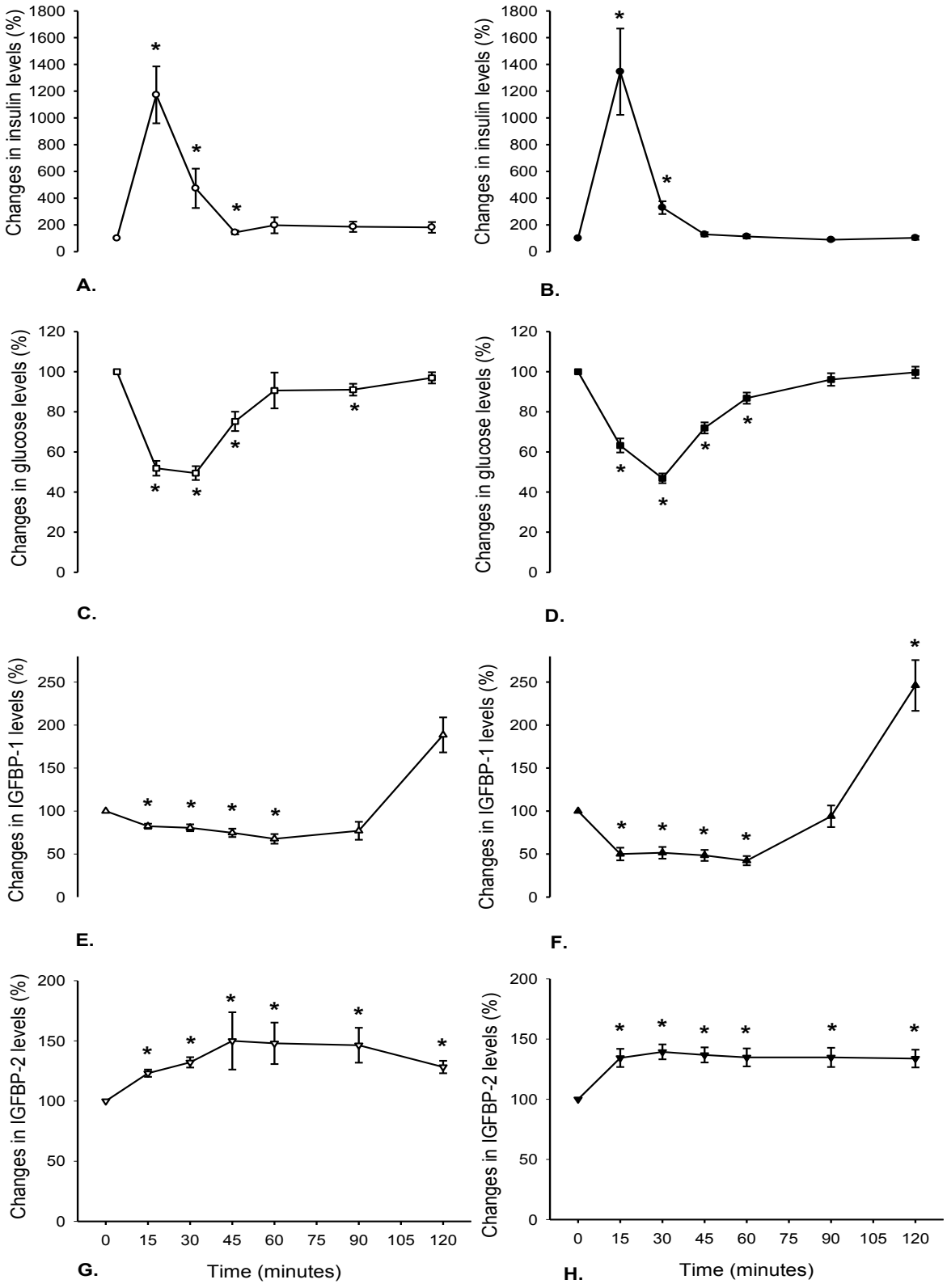


Figure 9. Mean (±SEM) changes in insulin **(A, B)**, glucose **(C, D)**, IGFBP-1 **(E, F)** and IGFBP-2 **(G, H)** concentrations after applying of insulin tolerance test in 13 lean participants (**A, C, E, G)** and

in 12 obese participants (**B, D, F, H)**. (* p < 0.01). All values are presented compared to the baseline value.

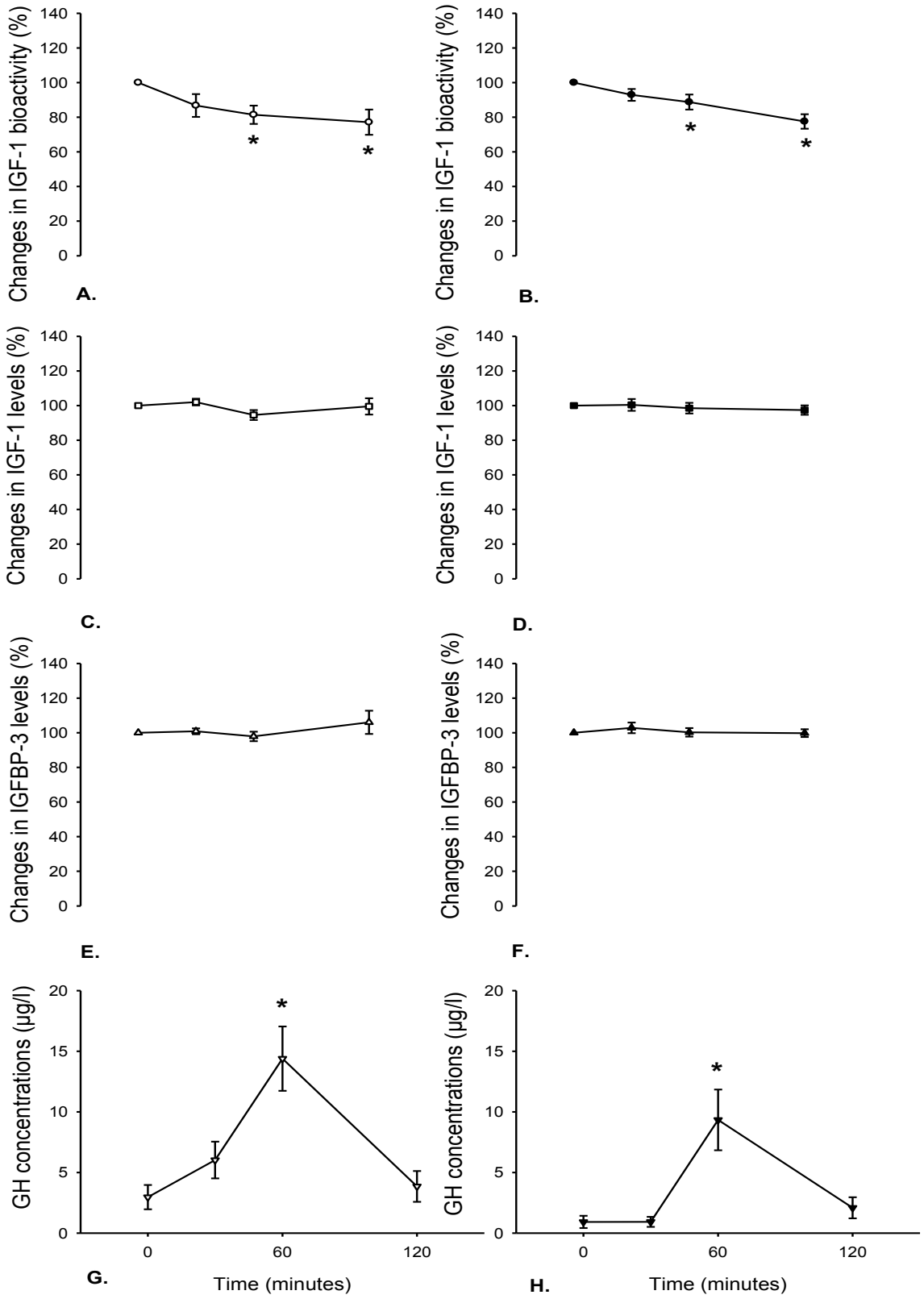


Figure 10. Mean (±SEM) changes in IGF-1 bioactivity **(A, B)**, total IGF-1 **(C, D)**, IGFBP-3 **(E, F)** and GH **(G, H)** concentrations after applying of insulin tolerance test in 13 lean participants (**A, C, E,**

G) and in 12 obese participants (**B, D, F, H)**. (* $p < 0.01$). All values are presented compared to the baseline value.

4.2.2 In vitro study

The impact of insulin on IGF-1, IGFBP-1, IGFBP-2 and IGFBP-3 gene expression in mouse primary hepatocytes

As expected insulin had no direct effect on IGF-1 mRNA expression in mouse primary hepatocytes, whereas growth hormone significantly induced 4-fold increase in IGF-1 mRNA at a concentration of 300 ng/ml **(Fig. 11 A)**. Treatment of mouse primary hepatocytes with 100 nM insulin for 24 hours decreased both of IGFBP-1 mRNA and IGFBP-3 mRNA significantly by approximately 25% and 21% of the control levels respectively **(Fig. 11 B, C)**. Addition of 300 ng/ml GH induced more decrease in IGFBP-1 mRNA and attenuated the decrease in IGFBP-3 mRNA significantly **(Fig. 11 B, C)**. Insulin induced a significant 6-fold increase in IGFBP-2 mRNA **(Fig. 11 D)**. GH tended to attenuate the insulin-induced transcription of IGFBP-2, although this effect was not statistically significant **(Fig. 11 D).**

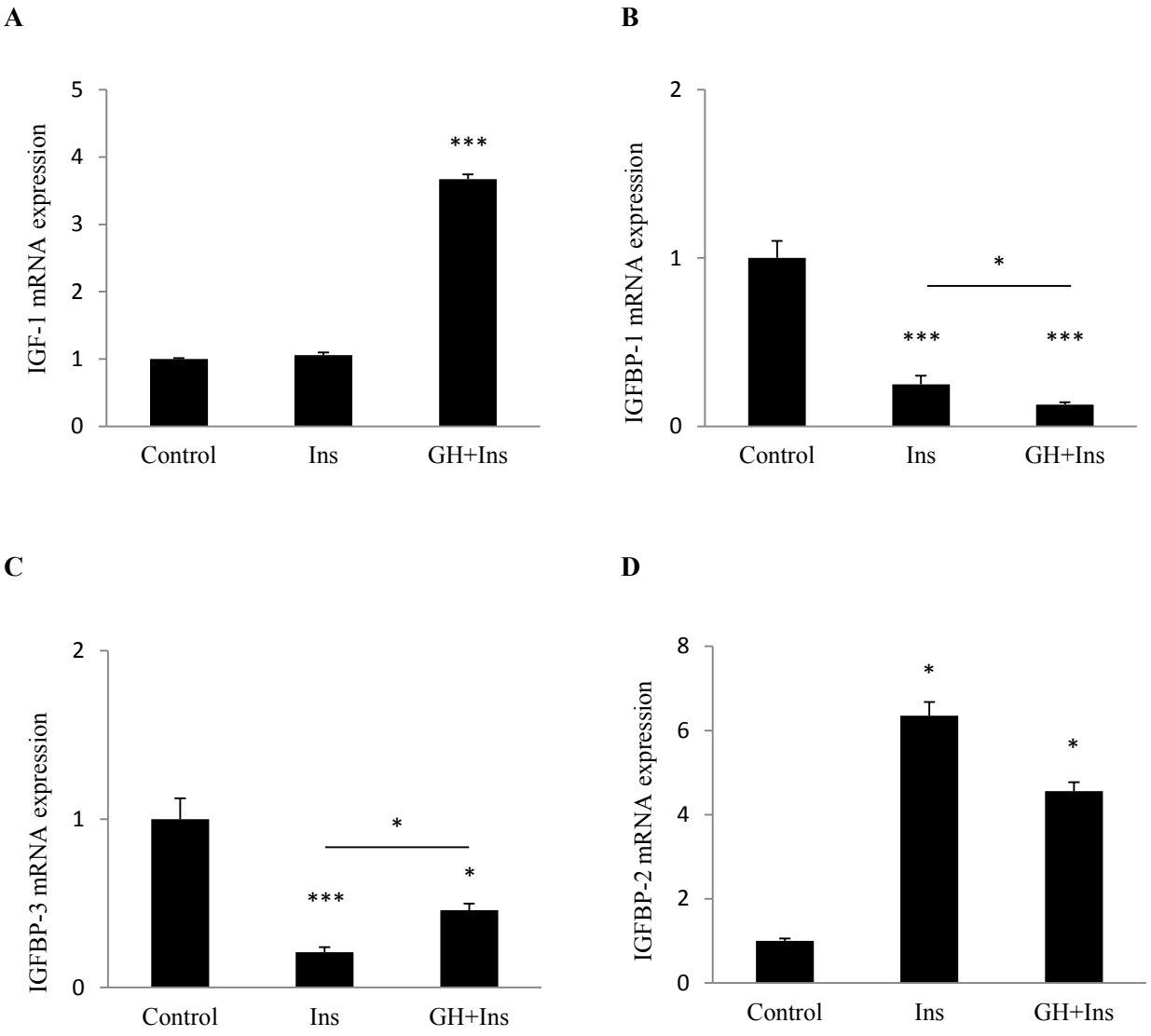


Figure 11. Relative changes of IGF-1 **(A)**, IGFBP-3 **(B)**, IGFBP-1 **(C)** and IGFBP-2 **(D)** mRNA expression in mouse primary hepatocytes after treatment with insulin in the presence or absence of GH. Bars show means ± SEM of four independent experiments done in duplicate. * $P < 0.05$, ** $P< 0.01$, *** $P< 0.001$**.**

5. Discussion

5.1 Glucagon administration study

This study is the first one focusing on the interaction between glucagon and IGF-1 bioactivity as a possible mechanism for glucagon-induced GH release. Previously published studies postulated that the glucagon-induced surge in GH secretion might result from the fall in the plasma glucose concentrations after the initial increase during the test, or through the glucagon-induced increase in noradrenaline concentrations and its interaction with α-receptors in the pituitary gland (127). According to this study, glucagon decreases IGF-1 bioactivity suggesting a new mechanism by which glucagon may impact GH release. It depends on the regulatory feedback mechanisms of decreased IGF-1 bioactivity on growth hormone gene expression and hormone release at both of the pituitary gland and the hypothalamus level. This effect was observed in lean and obese participants and preserved in T1DM patients indicating an insulin-independent effect.

However, Glucagon is known to be a counter-regulatory hormone of insulin involved in catabolic pathways associated with increased blood glucose levels such as gluconeogenesis, inhibition of glycogenesis and lipid storage. IGF-1 has similar effects as insulin in mediating the anabolic pathways, therefore decreased IGF-1 signaling by glucagon is expected to maintain glucose homeostasis.

The mechanism underlying glucagon-induced reduction in IGF1 bioactivity

Activation of IGF-1 signaling is determined by the presence and interplay between IGF-binding proteins such as IGFBP-1, IGFBP-2 and IGFBP-3 and IGF-1. These binding proteins prevent the IGF-1 from interacting with its receptor (128). Various studies have demonstrated that IGFBPs levels are regulated by different hormonal, nutritional and biological conditions like exercise, surgery, pregnancy and age (129-131). Therefore, free or unbound IGF-1 reflects IGF-1 bioactivity, the ability of IGF-1 to induce phosphorylation of its receptor (132). However, my *in vitro* experiment showed, in consistence with Firth and Baxter (133) that increased IGFBP-1,

IGFBP-2 and IGFBP-3 reduce IGF-1 bioactivity, determined by the KIRA method. In this study, the observed decrease in IGF-1 bioactivity after glucagon administration was not due to any changes in total IGF-I or IGFBP-3 concentrations, whereas it was related to a remarkable increase in serum IGFBP-1 and IGFBP-2 levels. Interestingly, glucagon was able to up-regulate basal and GH-stimulated IGF-1 transcript significantly, an effect that we did not see at the protein level in humans. Previously *in vitro* study showed that glucagon stimulated the basal and GH-induced IGF-2 in salmon hepatocytes at supraphysiological concentrations (134). Glucagon-like peptide 2, a transciption product of proglucagon gene induced IGF-1 mRNA in *in vitro* intestinal cultures and *in vivo* mouse small intestine (135). The difference between the effect of glucagon at the transcriptional level and its effects at the protein levels of IGF-1 could be due to the post-transcriptional modification or to the higher glucagon dose used *in vitro* compared with the physiological concentration. On the other hand, GH increases IGFBP-3 level (136) and glucagon-induced decrease in transcription of IGFBP-3 observed in mouse primary hepatocytes was reversed *in vivo*, most likely by the stimulated GH serum concentrations. IGF-1/IGFBP-1 ratio has been considered as a potential predictor of IGF-1 bioactivity (137). In this study, glucagon increased serum IGFBP-1 concentrations in all tested groups and tended to increase IGFBP-1 mRNA in mouse primary hepatocytes. Consistently, Hilding *et al.* reported the same induction effect of glucagon on IGFBP-1 secretion *in vitro* and *in vivo*, despite the lack of detectable changes at the transcriptional level (138;139). IGFBP-1 is regulated mainly by the nuclear translocation of proteins called Forkhead O transcription factors (FOXO) that bind to the IGFBP-1 promoter and increase its expression. It has been reported that insulin decreases IGFBP-1 through the PI3K/Akt/FOXO pathway (72). However, Mounier *et al.* reported that the insulin-induced decrease in IGFBP-1 expression may even occur FOXO-independently by the activation of mTOR (140;141). Nevertheless, FOXO activity and its nuclear-cytoplasmic shuttling is determined, in addition to its PI3K/Akt pathway-mediated phosphorylation/dephosphorylation, by its acetylation/deacetylation state controlled by multiple factors like the silent information regulator 2 (Sir2) mammalian orthologue sirtuin 1 (SIRT1) and histone deacetylases class IIa (IIa HDACs) (142;143). Moreover, activation of class I/IIa HDACs by glucagon that deacetylates and activates FOXO has been reported (144). However, the glucagon induced a slight increase in IGFBP-1 mRNA expression in mouse primary hepatocytes was independent of Akt phosphorylation. Hence, the mechanism underlying glucagon-induced IGFBP-1 secretion need more investigations.

IGFBP-2 is another metabolically-regulated modulator of IGF-1 bioactivity (108). Arafat *et al.* showed that insulin reduces IGF-1 bioactivity by up-regulation of IGFBP-2 (108). Li *et al.* reported that insulin regulates IGFBP-2 expression through PI3K/mTOR pathway (110). In agreement with this, these results indicate that glucagon increases IGFBP-2 independent of changes in circulating insulin concentrations. Although I could not detect a statistically significant increase in IGFBP-2 gene expression, levels were numerically higher after glucagon stimulation suggesting an impact of glucagon on IGFBP-2 expression.

Mothe-Satney *et al.* found that glucagon increases mTOR phosphorylation on Serine 2448, whereas it inhibits the phosphorylation of its downstream targets suggesting the involvement of this phosphorylation site of mTOR in other unknown functions (145). In addition, some studies suggest that glucagon exerts some of its effects by induction of PI3Kinase pathway (146;147). Thus, it can be postulated that the glucagon-induced up-regulation of IGFBP-2 secretion is caused by the glucagon-induced modulation of PI3kinase signaling and the consequent activation of mTOR, despite more analysis is required.

Although the positive effects of high protein diet on weight control have been previously suggested, the increased incidence of cardiovascular disease related to the consumption of this diet has been demonstrated (112-115). However, changes in GH/IGF-1 axis after high protein diet have been reported (148).

The relationship between GH/IGF-1 axis and mortality has been elucidated. GH deficiency (149), increased GH in acromegaly (150), increased IGFBP-1 level (151) and IGFBP-2 (152) have been reported in association with higher mortality rate.

Moreover, the correlation between reduced IGF-1 signaling and the development of diseases such as metabolic syndrome, hepatic steatosis, diabetes mellitus type 2, chronic kidney disease and hypertension have been evaluated (90-93). In addition, IGF-1 signaling is involved in the expression of genes such as Glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H dehydrogenase (quinone) 1 (NQO1) and heme oxygenase (decycling) 1 (HMOX1) which are responsible for the antioxidant defenses in the vessels (88;89). The present study speculates that the negative effects of high protein diet, characterized by increased in glucagon secretion, in association with increased risk of cardiovascular disease and mortality rate, is at least partly

due to the ability of glucagon to decrease bioactive IGF-1, a critical protector of cardiovascular vessels.

5.2 Insulin-induced hypoglycaemia study

Accumulating evidence suggests that glucose interferes with GH/IGF-1 system (116;117). In addition, increased fasting blood glucose levels related to insulin resistance are associated with increased IGFBP-1, decreased IGFBP-2 and increased IGF-1 bioactivity (108). Despite the above mentioned data, this study demonstrated herein that the insulin-induced decrease in IGF-1 bioactivity under hypoglycemic condition is very similar to that shown in euglycemic state (108). Nevertheless, this investigation reveals that the acute changes in blood glucose levels play no role in insulin-GH/IGF-1 system interaction. This finding is supported by the work of Ma *et al.* showing that insulin analogues used to treat type 1 diabetes mellitus have the same impact on IGF-1 bioactivity compared with human insulin, despite of their different glucose-lowering abilities (153).

As described before, IGF-1 bioactivity is determined by the interplay between GH, total IGF-1 and the inhibitory and stimulatory IGF binding proteins. It reflects the active free IGF-1 fraction that induces IGF-1 signaling. I did not observe any change in total IGF-1 concentrations during insulin-induced hypoglycemia in all participants which excludes its effect on decreased IGF-1 bioactivity, a result which was previously obtained under euglycemic condition (108). However, some other previous studies indicated that hypoglycemia has no influence on IGF-1 secretion patterns (154;155). Conversely, neonates with hyperinsulinaemic hypoglycemia, a common heterogenous metabolic abnormality resulting from different genetic mutations of the pancreatic β-cells, show relatively low serum IGF-1 (156). Furthermore, foods with low glycemic load that decreases post-prandial responses of glucose are correlated with lower IGF-1 concentration (157). The variation in IGF-1 response between our results and the above mentioned studies could be explained by the different impact of long-term versus short-term hypoglycemia. Although my *in vitro* study showed that insulin decreased IGFBP-3 mRNA in mouse primary hepatocytes and the exposure to high glucose increased IGFBP-3 mRNA in a porcine PTEC cell line (LLC-PK1 cells) (158), we demonstrate in this study that IGFBP-3 secretion is not influenced by insulin, regardless of the glycemic state. Consistently, it is reported that IGFBP-3 levels do not change in response to glucose loads after applying the oral glucose tolerance tests (159). In addition, the presence of glucose does not affect the binding between IGF-1 and its most abundant circulating carrier protein (IGFBP-3), an index for IGF-1 bioactivity (160). Down-regulation of IGFBP-1 by insulin has

been evaluated, whereas hypoglycemia-dependent alteration of IGFBP-1 concentration has been suggested (161). Our data clarify that insulin decreases IGFBP-1 secretion under hypoglycemia. However, low serum IGFBP-1 levels are considered as a helpful marker to differentiate hyperinsulinism-induced hypoglycaemia from other hypoglycaemic disorders like hypopituitarism, fatty acid oxidation and glycogen storage disorders (162).

Treatment of nonislet cell tumor hypoglycaemia decreases serum IGFBP-2 indicating an inverse correlation with serum glucose (163). In contrast, hyperglycemia is associated with increased IGFBP-2 production as a result of glucose-enhanced acetylation of histones bound to IGFBP-2 promoter (164). In our study, insulin-induced up-regulation of IGFBP-2 was achieved under hypoglycemic state as under euglycemic state before. Thus the observed suppression of IGF-1 bioactivity in our investigation is explained as in Arafat *et al.* (165) by up-regulation of IGFBP-2. Insulin-induced hypoglycemia is a potent stimulus of GH secretion, although the underlying mechanism is not fully clear, especially by excluding any change occurred in GHRH and somatostatin secretion (166;167). Hence, herein this study suggests the hypoglycemia-induced decrease in IGF-1 bioactivity as a possible mechanism depending on the feedback effect on the pituitary gland level.

It is known that the occurrence of hypoglycemia, as a result of an intensive glucose control, increases the cardiovascular risk and the rate of mortality among diabetic patients. Despite various mechanisms including increased endothelial dysfunction, increased oxidative stress within mitochondria, induced platelet aggregation and activation of pro-inflammatory mechanisms have been suggested (168-170), the exact mechanism explaining hypoglycemia-induced higher death rate remains unclear. How changes in GH/IGF-1 axis and reduced IGF-1 signaling contribute to higher mortality rate have been well discussed in the previous session. This study showed that insulin-induced hypoglycemia decreases IGF-1 bioactivity in humans by up-regulation of IGFBP-2 suggesting a new mechanism underlying hypoglycemia-increased cardiovascular risk and mortality rate.

6. Conclusion

This work shows that glucagon is able to suppress bioactive IGF-1 levels independently of endogenous insulin concentrations. Moreover, it demonstrates that this reduction in IGF-1 bioactivity is caused by up-regulating the secretion of IGF-binding proteins IGFBP-1 and IGFBP-2. Furthermore, GH hyper-secretion after glucagon injection which is used as a GH stimulation test to evaluate an intact pituitary gland function or GH deficiency may be caused by a decreased IGF-1 bioactivity that exerts a feedback mechanism at the pituitary level. In addition, this study speculates that the negative effect of high protein diet, characterized by increased glucagon secretion, in association with increased risk of cardiovascular disease and mortality rate, is at least partly due to the ability of glucagon to reduce bioactive IGF-1, a critical protector of cardiovascular vessels. However, more analysis regarding the mechanism underlying glucagon-induced modulation of IGFBPs is required.

Although accumulating evidence suggests that glucose interferes with GH/IGF-1 system, this study shows, that the insulin-induced decrease in IGF-1 bioactivity under hypoglycemic condition is very similar to that shown in euglycemic state before. Nevertheless, the current investigation reveals that the acute changes in blood glucose level play no role in insulin-GH/IGF-1 system interaction. Moreover, the insulin-induced hypoglycemia decreases IGF-1 bioactivity through up-regulation of IGFBP-2. These results represent a possible and previously poorly explored mechanism explaining the strong association between hypoglycemia and increased cardiovascular mortality among diabetic patients.

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At last but not at least, I dedicate this thesis to my two lovely boys, Haidrah and Hamzah, who have given me so much strength and happiness. Without you in my life I could never have completed this study. I hope that I could have been a good mother for you throughout this difficult period.

List of publications and conferences

Publications

Bumke-Vogt C, Osterhoff MA, Borchert A, Gu man-Pere V, Sarem Z, Birkenfeld AL, B hr V, Pfeiffer AF. **The flavones apigenin and luteolin induce FOXO1 translocation but inhibit gluconeogenic and lipogenic gene expression in human cells.** PLoS One. 2014 Aug.

Sarem Z, Bumke-Vogt C, Assefa B, Weickert MO, Adamidou A, B hr V, Frystyk J, M hlig M, Spranger J, Lieske S, Birkenfeld AL, Pfeiffer AF and Arafat AM. **E GF-** , , . Submitted.

Conferences

Oral presentation: **Glucagon modulates the expression of IGF-1 system members in mouse primary hepatocytes** at 31th international symposium on DNSG 2013 in Dubrovnik, Croatia.

Poster presentation: **Glucagon modulates the expresseion of IGF-1 system members in mouse primary hepatocytes** at 10th NuGO week 2013 in Munich, Germany

Poster presentation: **Exogenous glucagon decreases IGF-I bioactivity in humans, independently of insulin levels, by modulating its binding proteins** at 50th EASD 2014 in Vienna, Austria.

Poster presenation: **Glucagon-IGF-1 bioactivity interaction: a link between high protein diet and cancer development** at 11th NuGO week 2014 in Neapel, Italy.

Poster presentation: **Glucagon decreases IGF-1 bioactivity in humans: a novel mechanism by which caloric restriction inhibits cancer development** at 58. Symposium der deutschen Gesellschaft für Endokrinologie (DGE) in Lübeck, Germany.

Poster presentation: **Insulin-induced hypoglycemia decreases IGF-I bioactivity in humans: a missing link to increased mortality in diabetic patients?** at ECE 2015 in Dublin, Ireland.

Thesis declaration

Hereby I certify that this thesis is a result of my own research and contains no material that have been used by another person except where appropriate reference or acknowledgements have been included.

Date Signature

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