REGULATION OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND IGF-BINDING PROTEIN-1 (IGFBP-1) mRNA LEVELS IN CULTURED RAT HEPATOCYTES

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Abbreviated form of the Title of the Thesis

Regulation of IGF-I and IGFBP-1 mRNA levels in Rat Hepatocytes.

This thesis is dedicated to my children

Aly and Farid

ABSTRACT

The liver is a major site of production of circulating levels of insulin-like growth factor-I (IGF-I) and IGF-binding proteins (IGFBPs). We have used primary cultured rat hepatocytes maintained under serum free conditions to explore the regulatory role of various hormones on hepatic IGF-I and IGFBP-1 mRNA levels.

IGF-I mRNA levels were stimulated 2.0 to 2.5 fold by bovine growth hormone (bGH) and 1.8 to 2.0 fold by glucagon but on combining bGH and glucagon, a synergistic effect was observed and IGF-I mRNA level was augmented 10 to 12 fold. Octreotide blocked the hGH induced stimulation of IGF-I production in serum and hepatic IGF-I mRNA levels in hypophysectomized rats. This effect could have been partly due to the low levels of glucagon in serum when hypophysectomized rats were treated with hGH and octreotide. Octreotide was also found to inhibit GH stimulated IGF-I mRNA levels in rat hepatocytes.

The unique synergy observed with glucagon and bGH on IGF-I mRNA levels in hepatocytes was not reproduced by T_3, oPRL, dexamethasone, EGF or insulin when each was added in combination with bGH or glucagon. Like glucagon, the additon of IBMX or $(Bu)_2$cAMP stimulated IGF-I mRNA levels 1.8 to 2.0 fold, but in the presence of bGH, IGF-I mRNA levels were stimulated 10 to 12 fold. PMA stimulated IGF-I mRNA levels 1.2 to 1.4 fold but displayed no synergism when added with bGH. The stimulatory effect of bGH plus glucagon on IGF-I mRNA levels was inhibited

in PKC depleted cells, in the presence of inhibitors of PKC and in the presence of cycloheximide. bGH had no posttranscriptional effect on IGF-I mRNA stability whereas glucagon or $(Bu)_2$cAMP stabilized IGF-I mRNA at a posttranscriptional level.

In summary, the major hormonal regulators of hepatic IGF-I mRNA levels appear to be GH and glucagon. Hepatic IGF-I mRNA levels are regulated by pathways involving protein kinase C and protein kinase A as well as by synthesis of one or more protein(s).

Glucagon and dexamethasone each stimulated IGFBP-1 mRNA levles 3 to 4 fold whereas bGH and T_3 each inhibited IGFBP-1 mRNA levels 45 to 70 %. Insulin, which inhibited IGFBP-1 mRNA levels 95 %, was the most powerful inhibitor and was also found to inhibit IGFBP-1 mRNA levels in the presence of dexamethasone. IBMX and $(Bu)_2$cAMP stimulated IGFBP-1 mRNA levels 6 to 8 fold whereas PMA inhibited IGFBP-1 mRNA levels 40 to 50 %. The inhibitory effect of bGH on IGFBP-1 mRNA levels was abolished in PKC depleted cells and also in the presence of inhibitors of PKC. In the presence of cycloheximide, IGFBP-1 mRNA was superinduced by bGH. bGH had no posttranscriptional effect on IGFBP-1 mRNA whereas glucagon and $(Bu)_2$cAMP stabilized IGFBP-1 mRNA at a postranscriptional level.

In summary, bGH, T_3 and insulin inhibited whereas dexamethasone and glucagon stimulated IGFBP-1 mRNA levels in hepatocytes. Effect of glucagon may be via elevation of cAMP

levels, whereas the effect of bGH may be via activation of PKC levels. The inhibitory effect of bGH appears to require synthesis of one or more protein(s) besides stimulation of PKC levels.

RESUME

Le foie est un site de production important du facteur de croissance insulinique de type I ("insulin-like growth factor-I", IGF-I) et des protéines liant les facteurs de croissance insuliniques ("IGF-binding proteins", IGFBPs). Nous avons utilisé des cultures primaires d'hépatocytes de rat maintenues dans des conditions exemptes de sérum dans le but d'étudier l'effet régulateur de différentes hormones sur les niveaux hépatiques d'ARN messager ARNm codant pour l'IGF-I et l'IGFBP-I.

Les niveaux d'ARNm de l'IGF-I augmentent de 2.0 à 2.5 fois en présence de bGH ("bovine growth hormone", hormone de croissance bovine) et de 1.8 à 2 fois en présence de glucagon, alors qu'en combinant la bGh et le glucagon, un effet synergique est obtenu, portant à de 10 à 12 fois l'augmentation des niveaux de base d'ARNm codant pour l'IGF-I. L'octréotide inhibe la stimulation de la production d'IGF-I induite par l'hormone de croissance humaine ("human growth hormone", hGH) dans le sérum, ainsi que les niveaux hépatiques d'ARNm de l'IGF-I chez les rats hypophysectomisés. Cet effet pourrait être partiellement attribuable aux bas niveaux sériques de glucagon chez les rats hypophysectomisés lors du traitement avec l'hGH et l'octréotide. L'octréotide peut également inhiber les niveaux d'ARN messager de l'IGF-I dans des hépatocytes de rat.

L'effet synergique observé avec le glucagon et la bGH, relativement à la stimulation des niveaux d'ANRm de l'IGF-I dans les hépatocytes n'a pas pu être reproduit par la T_3, l'oPRL (pro-

lactine ovine), la dexaméthasone, l'EGF ou l'insuline, lorsque chacun était utilisé en combinaison avec la bGH ou le glucagon. De façon similaire à celle du glucagon, l'addition d'IBMX ou de $(BU)_2$cAMP a fait augmenter les niveaux d'ARNm de l'IGF-I de 1.8 à 2.0 fois, alors qu'en présence de bGH, ces niveaux augmentaient de 10 à 12 fois. Le PMA a provoqué un accroissement des niveaux d'ARN messager de l'IGF-I de 1.2 à 1.4 fois, cependant qu'aucun effet synergique n'a été observé lorsque le PMA était ajouté en combinaison avec la bGH. L'effet stimulateur du glucagon et de la bGH sur les niveaux d'ARN messager de l'IGF-I était inhibé dans les cellules déplétés en PKC, en présence d'inhibiteurs de la PKC, et également en présence de cycloheximide (au niveau post-transcriptionnel) la bGH n'a pas d'effet sur la stabilité de l'ARNm de l'IGF-I, alors que le glucagon ou le $(BU)_2$cAMP stabilisent les niveaux de ce dernier.

En résumé, la GH et le glucagon semblent être les plus importants régulateurs hormonaux des niveaux hépatiques d'ARNm et l'IGF-I. Ces niveaux seraient contrôlés par des voies intracellulaires impliquant les protéines kinase A et C ainsi que la synthèse d'une ou de plus d'une protéine.

Le glucagon et la dexamethasone ont fait augmenter les niveaux d'ARNm de l'IGFBP-I de 3 à 4 fois, alors que la bGH et la T_3 ont provoqué une diminution de ces niveaux de 45 à 70%. L'insuline qui a provoqué une diminution des niveaux d'ARNm de l'IGFBP-1 de 95%, était le plus puissant inhibiteur, et a également pu faire décroître les niveaux d'ARNm de l'IGFBP-1 en présence de dexamethasone. L'IBMX et le $(BU)_2$cAMP ont provoqué l'accroisse-

ment des niveaux d'ARNm de l'IGFBP-1 de 6 à 8 fois alors que la PMA a fait décroître ces niveaux de 40 à 50%. L'effet inhibiteur de la bGH sur les niveaux d'ARNm de l'IGFBP-I était aboli dans des cellules déplétées en PKC et également en présence d'inhibiteurs de la PKC. En présence de cyclohéximide, l'ARNm de l'IGFBP-I est incitée par la bGH. Au niveau post-transcriptionnel, la bGH n'a pas d'effet sur l'ARNm de l'IGFBP-I, alors que le glucagon et le $(BU)_2$cAMP stabilisent les niveaux de ce dernier.

En résumé, la bGH, la T_3 et l'insuline ont fait diminuer alors que la dexaméthasone et le glucagon ont provoqué l'accroissement des niveaux d'ARNm de l'IGFBP-1 dans des hépatocytes. L'effet du glucagon pourrait être médié par l'élévation des niveaux d'AMPc alors que celui du glucagon serait dû à l'activation de la PKC. En plus de l'activation de la PKC, l'effet inhibiteur de la bGH semble requérir la synthèse d'une ou de plusieurs protéines.

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PKA	protein kinase A
PKC	protein kinase C
K	kilodalton
Kb	kilobases
PIs	isoelectric points
M_r	molecular weight
CSF	cerebrospinal fluid
CNS	central nervous system
AP-1	activator protein-1
AP-2	activator protein-2
cAMP	3', 5'- cyclic adenosine monophosphate
GH-R	growth hormone receptor
G-R	glucagon receptor
Tyr K	tyrosine kinase
PM	plasma membrane
IDDM	insulin dependent diabetes mellitus
STZ-D	streptozotosin induced diabetes
DAG	diacylglycerol
GAPDH	glutaraldehyde-3-phosphate dehydrogenase

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ABBREVIATIONS

Growth Factors

IGF-I	insulin like growth factor-I
IGF-II	insulin like growth factor-II
EGF	epidermal growth factor
TGF-α	transforming growth factor-α
FGF	fibroblast growth factor
b-FGF	basic fibroblast growth factor
PDGF	platelet derived growth factor

Hormones

GH	growth hormone
PRL	prolactin
T_3	3,5,3'-triiodothyronine
Ins	insulin
Dex	dexamethasone
GH-V	growth hormone-variant form
LH	luteinizing hormone
FSH	follicle-stimulating hormone
ACTH	adrenocorticotropin
TSH	thyrotropin
PGE_2	prostaglandin E_2
PTH	parathyroid hormone
CG	chorionic gonadotropin
G	glucagon

PL	placental lactogen
A-II	angiotensin-II

Prefixes to hormones

b	bovine
h	human
o	ovine
r	rat

Somatomedins

SM	somatomedin
SM-A	somatomedin-A
SM-B	somatomedin-B
SM-C	somatomedin-C
$NSILA_p$	nonsuppressible insulin like activity precipitable
$NSILA_s$	nonsuppressible insulin like activity soluble
MSA	multiple stimulating activity
ILA	insulin like activity

Amino acids

Ser	serine
Gly	glycine
Ala	alanine
Val	valine
Cys	cysteine
Asp	aspartic acid

RGD	arginine-glycine-aspartic acid

IGF-binding proteins

BPs	binding proteins
IGFBPs	insulin like growth factor binding proteins
IGFBP-1	insulin like growth factor binding protein-1
IGFBP-2	insulin like growth factor binding protein-2
IGFBP-3	insulin like growth factor binding protein-3
IGFBP-4	insulin like growth factor binding protein-4
IGFBP-5	insulin like growth factor binding protein-5
IGFBP-6	insulin like growth factor binding protein-6
AFBP	amniotic fluid binding protein
α_1-PEG	pregnancy associated endometrial protein α_1-globulin
PP12	placental protein 12

Chemicals and Buffers

EDTA	ethylenediamine tetraacetate
EGTA	ethyleneglycol-bis (ß-amino ethyl ether) -N,N,N,N'-tetraacetic acid
DMEM	dulbecco's modified Eagle's medium
F12	Ham's F12 nutrient mixture
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
FCS	fetal calf serum
MOPS	morpholinopropanesulfonic acid
SSPE	sodium chloride, sodium phosphate, EDTA

SDS	sodium dodecyl sulfate
SSC	sodium chloride, sodium citrate
IBMX	3-isobutyl-1-methyl xanthine
$(Bu)_2$cAMP	N^6,2-0-dibutyryl adenosine 3',5' cAMP
PMA	4ß-phorbol 12ß-myristate 13α-acetate
α-$PDCo_2$	4α-phorbol, 12, 13, didecanoate
H_7	1-(5-isoquinolinyl sulphonyl)-2-methyl peperazine
Endo F	endoglycosidase F
dCTP	deoxycytidine triphosphate
PAGE	polyacrylamide gel electrophoresis
BSA	bovine serum albumin
CYX	cycloheximide
Act. D	actinomycin D
$1,25(OH)_2$ Vit. D_3	1,25 dihydroxy vitamin D_3
M6P	mannose-6-phosphate

Others

mRNA	messenger ribonucleic acid
cDNA	complementary deoxyribonucleic acid
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
BW	body weight
Sc	subcutaneous
RIA	radioimmunoassay
UV	ultaviolet
IRI	immunoreactive insulin

PKA	protein kinase A
PKC	protein kinase C
K	kilodalton
Kb	kilobases
PIs	isoelectric points
M_r	molecular weight
CSF	cerebrospinal fluid
CNS	central nervous system
AP-1	activator protein-1
AP-2	activator protein-2
cAMP	3', 5'- cyclic adenosine monophosphate
GH-R	growth hormone receptor
G-R	glucagon receptor
Tyr K	tyrosine kinase
PM	plasma membrane
IDDM	insulin dependent diabetes mellitus
STZ-D	streptozotosin induced diabetes
DAG	diacylglycerol
GAPDH	glutaraldehyde-3-phosphate dehydrogenase

PREFACE

In accordance with the guidelines concerning thesis preparation, the candidate has taken the option of writing the experimental portions of this thesis (Chapters 2 to 6, inclusive) in the form of original papers. This provision reads as follows " The candidate has the option, subject to the approval of their Department, of including as part of the thesis the text, or duplicated text, of an original paper or papers. Manuscript-style thesis must still conform to all other requirements explained in the Guidelines concerning thesis preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g., in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review, and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion. It is acceptable for these to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. In such instances, connnecting texts are mandatory and suplementary explanatory material is always necessary. Photographs or other material which do not duplicate well must be included in their original

form.

While the inclusion of manuscript coauthored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and what extent, and supervisors must attest to the accuracy of the claims at the Ph.D. Oral Defence. Since the task ot the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of the authors perfectly clear."

This format has allowed the inclusion, as chapters in this thesis, two published papers and three manuscripts which will shortly be submitted for publication. Each of these chapters bears its own Abstract, Introduction, Materials and Methods, Results and Discussion sections. A common Abstract, Introduction and Review of Literature, Aim of the Present Study, Summary, General Discussion and Contributions to Knowledge have been added. References appear at the end of each chapter and are grouped in order of appearence in the text. The two published papers and three manuscripts are as follows:

Chapter 2: Kachra Z, Barash I, Yannopoulos C, Khan MN, Guyda HJ and Posner BI 1991

The Differeential Regulation by Glucagon and Growth Hormone of Insulin-Like Growth Factor (IGF-I) and IGF Binding Proteins in Cultured Rat Hepatocytes. Endocrinology 128:1723-1730

Chapter 3: Serri O, Brazeau P, Kachra Z and Posner B 1992 **Octreotide Inhibits Insulin-Like Growth Factor-I Hepatic Gene Expression in the Hypophysectomized Rat: Evidence for a Direct and Indirect Mechanism of Action. Endocrinology** 130:1816-1821

Chapter 4: Kachra Z and Posner BI
Protein kinase C and protein kinase A are necessary but not sufficient to mediate bGH and glucagon effect on IGF-I mRNA stimulation in rat hepatocytes.

Chapter 5: Kachra Z and Posner BI
A new protein(s) synthesis is required for bovine growth hormone and glucagon effect on IGF-I mRNA stimulation in rat hepatocytes.

Chapter 6: Kachra Z and Posner BI
Bovine growth hormone inhibits whereas glucagon stimulates IGFBP-1 mRNA levels in cultured rat hepatocytes.

For all chapters, except chapter 3, the candidate was responsible for the preparation of manuscripts. In chapter 2, the candidate performed all the experiments except the following:

(a) All the IGFBP(s) assays were performed by C. Yannopoulos (Figures: 4, 5, 6 and Table 3).

(b) Dr. H. Guyda designed and supervised the RIA for

quantitation of IGF-I in culture medium (Figure 1).

(c) Dr. I. Barash initially started the project and established some of the methods used in this study.

(d) Dr. M.N. Khan participated in helpful discussions.

In Chapter 3, the candidate performed the Northern blot analysis on rat liver samples (Figures 2 and 3) and also carried out in vitro experiments in cultured rat hepatocytes (Table 3). The candidate was responsible for writing the result section for the above figures and table.

In Chapters 4, 5 and 6, the candidate was responsible for all the experiments performed.

All the experiments performed by the candidate were carried out in the Polypeptide Hormone Laboratory under the guidance and supervision of Dr. Barry Posner.

CHAPTER 1

REVIEW OF LITERATURE

I INSULIN-LIKE GROWTH FACTORS (IGFS) OR SOMATOMEDINS

A. HISTORICAL CONSIDERATIONS

The somatomedins or insulin-like growth factors (IGFs) constitute a family of growth hormone-dependent peptides with anabolic and mitogenic properties for a wide variety of cell lines (1). Originally these were recognized as growth hormone induced factor(s) that stimulated [^{35}S]sulfate incorporation into proteoglycans of rat cartilage (2). It is now apparent that these peptides are involved in diverse metabolic activities, including glucose and amino acid transport, protein synthesis, DNA and RNA synthesis and cell replication (3,4).

(a) The Sulfation Factor Hypothesis

In 1957, Salmon and Daughaday (2) first showed that the ability of serum from hypophysectomized rats to stimulate [^{35}S] sulfate incorporation into rat chondrocyte proteoglycans could not be restored by the in vitro addition of growth hormone. On the other hand, serum from hypophysectomized rats treated with growth hormone in vivo was capable of stimulating [^{35}S] sulfate uptake, this demonstrated the existence of a growth hormone-dependent anabolic substance which was called " Sulfation Factor".

Various terms have been used to describe the intermediate substance(s) involved in growth hormone action. A summary of these terms should be helpful in understanding the background to the evolution of thinking in this field. The sulfation factor purified from plasma and other sources was found to

stimulate thymidine incorporation into DNA and uridine incorporation into RNA. So this factor was also known as "Thymidine" factor (4).

(b) **Somatomedins**

In 1972, the term "Sulfation factor" was replaced by the term "Somatomedin" because of its broad anabolic and mitogenic properties (5). A peptide could be regarded as a "Somatomedin" if it possesses the following properties:

(i) Its concentration in serum must be growth hormone dependent.

(ii) It must promote the incorporation of sulfate into proteoglycans of cartilage.

(iii) It must be capable of stimulating DNA synthesis and cell multiplication.

Subsequent purification procedures yielded three distinct fractions, a basic peptide (SM-C) (1), a neutral peptide (SM-A) (6) and an acidic peptide (SM-B) (7). SM-B is no longer considered a true somatomedin as it does not stimulate [^{35}S]sulfate uptake into cartilage.

The concurrent study on insulin activity as measured in rat muscle and adipose tissue bioassays demonstrated that only a minor fraction of the insulin-like action of normal serum could be neutralized by the addition of anti-insulin antibodies (8). The remaining activity was termed nonsuppressible insulin-like activity (NSILA). Subsequent investigation demonstrated that

NSILA consisted of atleast three components, which were partially resolvable on the basis of their solubillity in acid-ethanol. Two low molecular weight (7000) soluble forms, termed nonsuppressible insulin like activity, soluble form, $NSILA_s$-I and $NSILA_s$-II, and a high molecular weight (90,000) acid-ethanol precipitable form, termed nonsuppressible insulin like activity precipitable form, $NSILA_p$ (9). In contrast to $NSILA_s$, $NSILA_p$ has no sulfation activity in vitro and was not considered relevant to the somatomedin family.

A third line of research at this time focused on isolating mitogenic factors from bovine serum and from serum-free medium conditioned by BRL-3A cells, a line of fetal Buffalo rat liver cells (10). This factor(s) isolated was termed multiplication-stimulating activity (MSA).

The acid ethanol treatment of serum has been shown to be effective in extracting SM as well as ILA from serum or Cohn fraction of serum (11). The NSILA was shown to have in vitro growth promoting activities similar to SM (12). MSA stimulated thymidine incorporation into DNA of chick embryo fibroblasts. It was shown that SM, ILAs and MSA were similar peptides.

(c) **Insulin-like growth factors (IGFs)**

In 1978, Rinderknecht and Humbel (13,14) succeeded in purifying and sequencing two somatomedin peptides from an acetone powder of a fraction from human plasma obtained by modified Cohn fraction. The acid-ethanol extract of this fraction was found to contain nonsuppressible insulin-like

activity (NSILAs) of a molecular weight between 6000 and 10,000 and was further purified by Sephadex G-75 and G-50 chromatography in acetic acid, polyacrylamide gel electrophoresis in sodium dodecyl sulfate at pH 8.8, chromatography on SE-sephadex, and polyacrylamide gel electrophoresis at pH 4.3. The purified peptides were renamed insulin-like growth factors (IGF) I and II. These peptides were reduced and carboxymethylated with iodoactate, and tryptic peptides were generated using α-chymotrypsin and trypsin. These peptides were further digested with proteases and carboxy and aminoterminal of intact proteins and of isolated peptides were determined. Sequential Edman degradation of peptides were performed using Beckmann Sequencer. Amino acid analysis was also performed on hydrolyzed samples.

Klapper et al (15) showed that amino acid of somatomedin-C purified from Cohn fraction IV of human plasma is identical to amino acid sequence of IGF-I and the same appears likely for the basic somatomedin described by Bala and Bhaumick (16). SM-A and the insulin-like activity (ILA) described by Posner et al (17) represent a mixture of IGF-I and IGF-II (18).

B. **STRUCTURE**

(a) **Human IGFs**

IGF-I is a basic peptide of moleucular weight 7649 and consists of 70 amino acids, while IGF-II is a neutral peptide of molecular weight 7471 and has 67 amino acids. IGF-I and -II

have been mapped to the long arm of human chromosome 12 and the short arm of human chromosome 11 respectively (19,20). Both growth factors are single-chain polypeptides with three intrachain disulfide bridges; 45 out of 70 amino acid positions in IGF-I and IGF-II are identical. They possess a striking structural similarity to human proinsulin, being composed of A- and B- chain regions with a connecting C peptide. A significant sequence identity exists between proinsulin and the IGFs, it is confined to the A and B chains. The C-peptide region of IGF-I and IGF-II is 12 and 8 amino acids long respectively, considerably shorter than 35 amino acid C-peptide of human proinsulin. Furthermore, the IGFs possess a unique carboxy-terminal extension, or D-peptide region (8 residues for IGF-I and 6 residues for IGF-II) which is absent in proinsulin. In total of the 51 amino acids of insulin, 20 are identical in IGF-I and IGF-II, including all the disulfide bond-forming cysteines.

Blundell et al (21) have proposed a model for the tertiary structure of IGF-I based on the striking sequence homology with insulin and proinsulin. This model accounts for the following phenomena:

(a) The failure of IGF-I to react with antibodies against insulin.

(b) The failure of insulin and proinsulin to react with antibodies against IGF-I.

(c) The weak cross-reactivity of insulin and IGF-I with each other's receptors.

(d) The inability of insulin and proinsulin to compete for occupancy of the IGF carrier proteins.

It has been suggested that cross-reactivity in various receptor assays may be accounted for by partial structural homology in the surface regions of the A and B chains, while the distinct antigenicity results from the nonhomologous C-peptide regions and the unique D-peptide.

The striking structural homologies between insulin and IGF explain the observation that somatomedins have insulin-like activity, while insulin has mitogenic activity in a variety of cell lines. It appears likely that these peptides have evolved from a common ancestor protein.

(b) **Rat and human IGFs**

The complete sequence of rat IGF-I was deduced from the nucleotide sequence of the rat IGF-I gene by Shimatsu and Rotwein (22). Only three amino acid differences were noted between rat IGF-I and human IGF-I, a proline for aspartic acid at position B 20, and isoleucine for serine at position C 35 and threonine for alanine at position D 67.

Rat IGF-II (MSA) was isolated from conditioned medium of the Buffalo rat hepatocytes cell line by Marquardt et al (23) and amino acid sequence was determined. The sequence of rat IGF-II has only five substitutions compared to human IGF-II including Ser for Gly at position 22, Ser for Ala at position 32, Gly for Ser at position 33, Ala for Val at position 35 and Asp for Ser

at position 36.

Mammalian IGF structures are highly conserved. All the IGF-I peptides contain 70 amino acids and all the IGF-II peptides contain 66 or 67 amino acids. The substitutions are relatively few and conservative in nature.

C. BIOSYNTHESIS

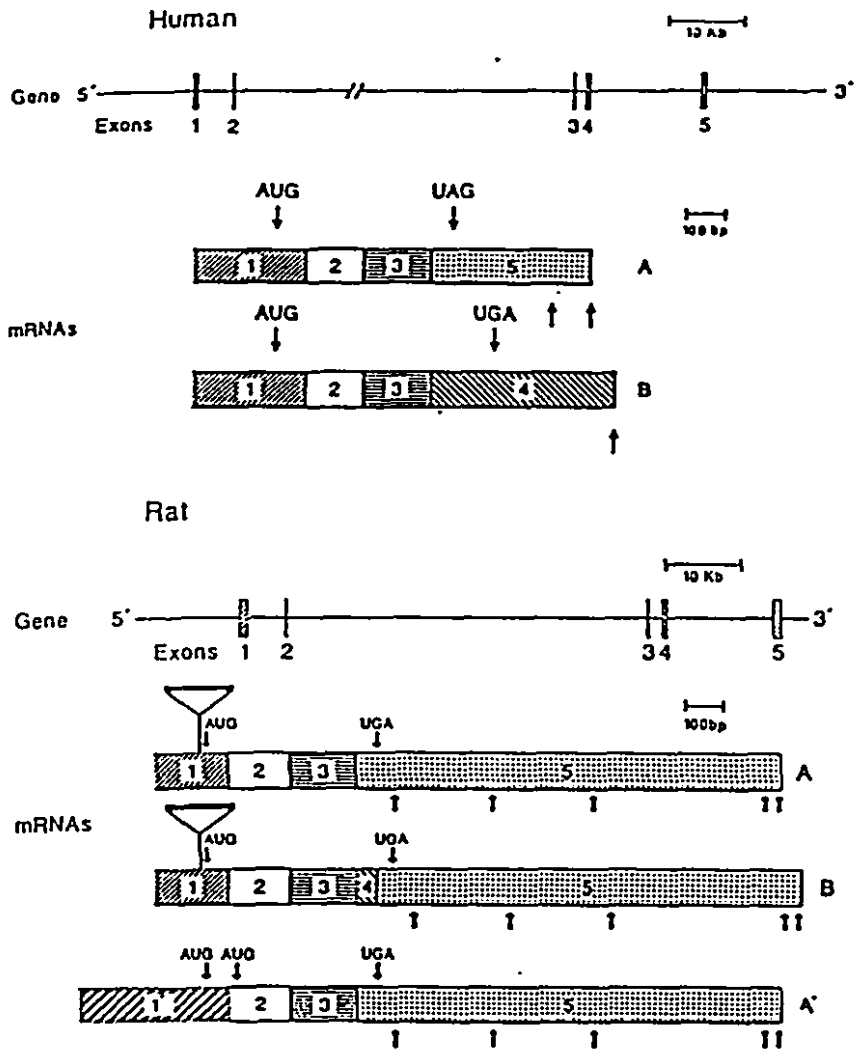
(a) IGF-I gene and mRNA structure

IGF-I and IGF-II genes from both human and rat have been characterized (19,24-29). The human and rat IGF-I genes (Figure 1) (24), contain atleast 5 exons, separated by 4 introns ranging in size from 1.9 to 50 kilobases (kb). All 5 exons contain some coding information. Exons 1 and 2 encode portions of the signal peptides; exons 2 and 3 contain the mature IGF-I molecule; while exon 3 also encodes the proximal portion of the E domain; and exons 4 and 5 contain the E regions of IGF-IB and IGF-IA respectively, as well as 3' untranslated sequence (24,27). The human IGF-I gene is transcribed and processed into 2 types of mRNAs. IGF-IA which comprises exons 1, 2, 3 and 5 and encodes 153 amino acids and IGF-IB which contains exons 1, 2, 3 and 4 and encodes 195 residues (30-32). IGF-IA mRNA appears to have 2 distinct polyadenylation sites, while IGF-IB mRNA has a single poly (A) addition sequence (30,31,33).

In the rat, processing of the primary IGF-I gene transcript appears to be more complicated than in the human. Alternative mRNA splicing occurs at both the 5' and 3' ends of the gene and there are at least 5 functional polyadenylation sites at the 3'

Figure 1

IGF-I Gene and mRNA Structure



Structure and expression of the IGF-I gene. The organization of the genes encoding human and rat IGF-I is depicted. Exons are numbered 1 through 5. Indicated *below each gene* is the pattern of alternative RNA splicing that generates the different mRNA transcripts. IGF-IA and IGF-IB mRNAs are noted. The *stippled region* in the 5'-end of the rat mRNA marked A' has not yet been positioned on the genomic map. The 5'-end of each mRNA is left open since it has not been defined. The beginning and end of the coding region for each mRNA is marked by AUG and UGA/UAG, respectively. Possible alternative polyadenylation sites are indicated by unlabeled arrows.
Taken from reference 24.

end of exon 5 (24,34-38). The most abundantly expressed 5' untranslated region can contain either an insertion or deletion of a 186 nucleotide fragment (37). Rat IGF-I gene transcripts have also been shown to contain three alternative 5' untranslated regions (38).

Processing of IGF-I gene transcript also occurs at the 3' end. There are at least 5 polyadenylation sites within exon 5, generating mRNAs with 3' untranslated regions of variable lengths (36,38). Rat and mouse IGF-I mRNAs (containing exons 1, 2, 3 and 5) are very similar to human IGF-IA. 17/19 amino acids are identical among the three species within the part of E domain specific to IGF-IA precursor. Rodent IGF-IB mRNA is generated by the addition of a 52 nucleotide "mini-exon" during processing of the primary rat or mouse IGF-I gene transcript (33,36,39). This mini exon is spliced between exons 3 and 5 and leads to a change in the translational reading frame. The 159 amino acid rodent IGF-IB precursor peptide thus differs substantally at its carboxy-terminus from human protein (31).

Chicken (40) and human (41) IGF-I gene promoters have recently been characterized. There may be two promoters present in the human IGF-I gene, one adjacent to exon 1 and the other adjacent to exon 2.The human IGF-I gene promoter has been shown to lacks TAATA box (41). There are multiple transcription start sites for human (41) and rat (42) IGF-I genes. The presence of two promoters has also been predicted for the rat IGF-I gene (42).

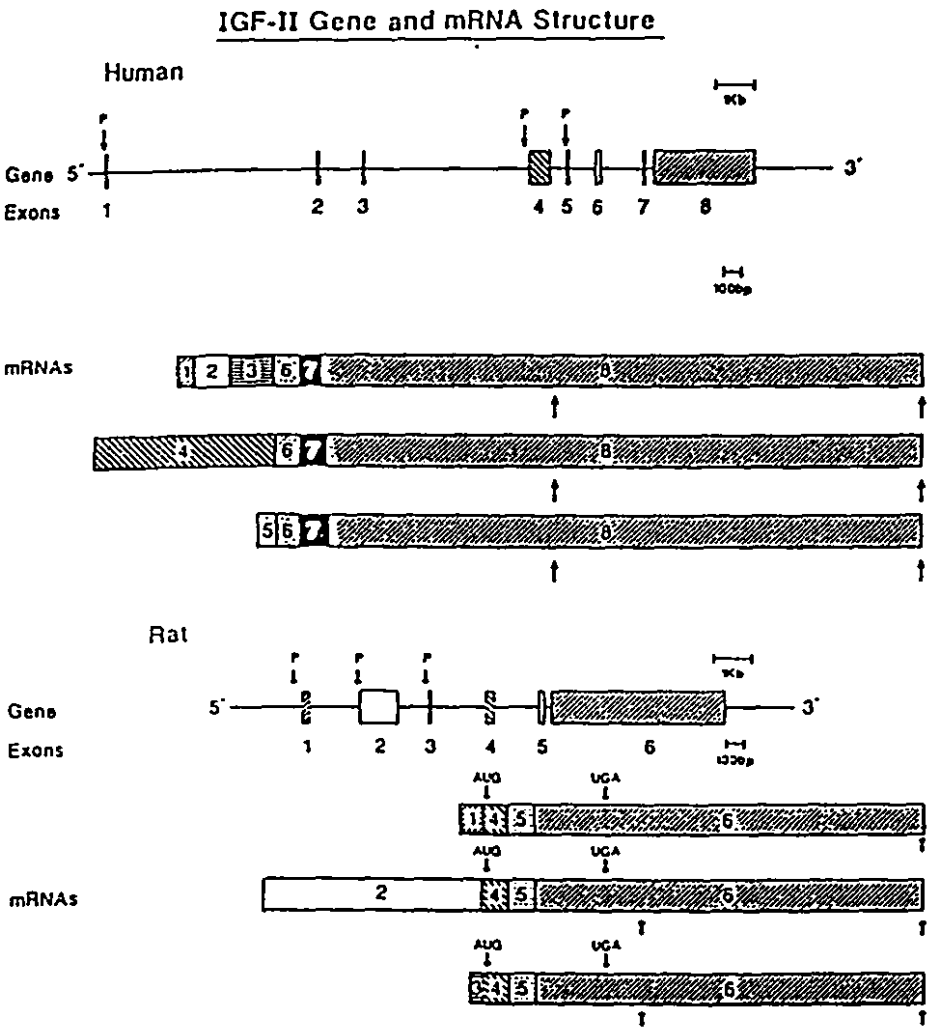
Several bands are detected on Northern blots of

polyadenylated mRNA from rat, mouse or human tissues after hybridization with an IGF-I probe. These bands range in size from 0.7 to more than 7.0 kb (31,34,35,39,). The major transcripts are 7.0 kb, 1.8 kb and 0.7 to 1.1 kb. Multiple IGF-I mRNA transcripts may be generated by alternative splicing of exons 4 and 5 (34), use of multiple transcription initiation sites (42), use of different promoters (41) and variable polyadenylation (43).The mRNA species of 0.7-1.1 kb consist of IGF-IA mRNA and that at 1.8 kb consists of IGF-IB mRNA. 7.0 kb mRNA was found to contain all the sequences present in 1.1 kb IGF-IA mRNA and in addition an unusually long 3' terminal untranslated sequence of over 6.0 kb. Two alternately used poly (A) addition sites within exon 5 of IGF-I gene give rise to 1.1 kb and 7.0 kb mRNA species (44).

(b) **IGF-II gene and mRNA structure**

As shown in Figure 2 (24), human IGF-II gene is composed of eight exons distributed over nearly 30 kb DNA and contains three promoters. The eight human IGF-II exons are transcribed and processed into three different mRNA species that share common coding and 3'-untranslated regions (exons 6-8) but differe in their 5'-untranslated sequences (26,19,45-48). These mRNAs arise as a consequence of the use of different promoters and by alternative RNA splicing (Figure 2). Transcription from the most upstream 5'-promoter leads to an mRNA of 5.3 kb containing three untranslated exons with the coding block,

Figure 2



Structure and expression of the IGF-II gene. The organization of the genes encoding human and rat IGF-II is depicted. Exons are numbered 1 through 8 for human IGF-II and 1 through 6 for rat IGF-II. Alternative promoters are designated with the letter P. Indicated *below each gene* are the mRNAs that result from transcription using the different promoters. The coding regions of the mRNAs are marked by AUG at the beginning and UGA at the end. Possible alternative polyadenylation sites are noted by *unlabeled arrows*.

Taken from reference 24.

while use of the other two promoters leads to mRNAs of 6.0 and 4.9 kb respectively, each containing a single untranslated exon at the 5'-end.

The rat IGF-II gene also contains three coding exons and has three promoters (Figure 2) (24). The structure and the sequence of two of the promoters and their corresponding noncoding exons are conserved between rodents and humans (19,28,29,45). The most 5'upstream rat IGF-II promoter (49) differs from its human counterpart in relative location, nucleotide sequence and is associated with a single untranslated exon.

Multiple RNA species ranging in size from 6.0 to 1.2 kb have been detected on Northern blots from rat tissues (28,29,49-52). The 5.0 kb mRNA is derived from the middle promoter and 4.0 kb species is derived from the most 3' promoter. The other species may be formed by variable RNA processing (Figure 3) (53) or polyadenylation at the 3'-end of the gene. The exact mechanisms involved in the synthesis of these RNA species have not been fully determined.

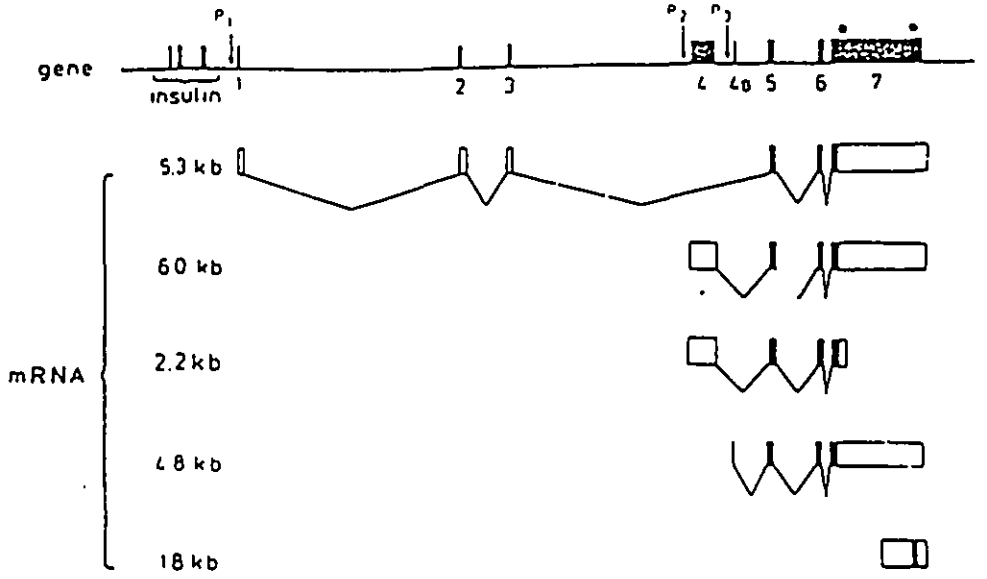
(c) **IGF precursor peptides**

The precursor structure of human, rat, mouse, pig. salmon and chicken IGF-I and human, rat and mouse IGF-II have been determined (30,45,54-56).

Two different human IGF-I precursor peptides have been predicted from cDNA cloning studies (30,31). The primary translation product of IGF-IA mRNA contains 153 amino acids,

Figure 3

Human IGF-II gene and mRNA transcripts



Schematic representation of the human IGF-II gene and the various mRNA transcripts. The human IGF-II gene is located very close to the insulin gene on chromosome 11. The gene consists of 8 exons, named 1 - 4, 4B and 5 - 7. The coding regions are indicated as black boxes in the mRNAs. Polyadenylation sites are marked by asterisks. Taken from reference 53.

and that of IGF-IB mRNA 195 residues. The amino acid sequences of these two proteins are identical for the initial 134 residues: including the signal peptide [amino acids 1-48 (32)], the 70 residue IGF-I molecule [amino acids 49-118], and 16 amino acids comprising the initial part of the E domain (Figure 2). The IGF-IA precursor contains an additional 19 residues, and IGF-IB, 61 (31). The 2 mRNAs encoding the A and B pre-pro IGF-I peptides arise via alternative splicing of the IGF-I gene. Messenger RNAs encoding similar IGF-IA and IGF-IB precursors also have been identified by cDNA cloning in the rat and mouse (34-37,39) and an IGF-IA homolog has been cloned from porcine liver (54).

Forty-three of the 48 amino-terminal amino acids comprising the putative signal peptide (32) are identical between human and rat IGF-I. The carboxy-terminal 35 amino acids of IGF-IA are highly conserved with only 5 differences among human, rat, mouse and pig precursors (54). Only the carboxy terminus of the IGF-IB precursor diverges between species. Fewer than 50% of residues are identical between human and rat or mouse (34-37,39), a probable result of the different mechanisms by which IGF-IB mRNAs are generated in these three species.

The amino acid sequence of pre-pro-IGF-II has been determined in human, rat and mouse (30,46-49,56). The primary IGF-II translation product contains 180 residues, including 24 residue amino-terminal signal peptide, a 67-residue IGF-II molecule, and an 89 residue carboxy-terminal E domain. There is strong sequence conservation between species. In the 67 residue

IGF-II molecule, 61/67 aminoacids are identical and in amino-terminal signal peptide, 20/24 are identical and in the carboxy-terminal E peptide 68/89 are identical in human and rodent. In both growth factor precursors, there is a single arginine residue at the first position after the carboxyl end of the mature protein, suggesting that a cleavage enzyme with a monobasic recognition site is involved in posttranslational proteolytic processing.

D. PRODUCTION

Endocrine and autocrine/paracrine mode of production

Liver has been shown to be the major source of production of IGFs (57,58). The mode of synthesis and secretion of IGF by liver has been studied in perfused rat liver (59), in liver explants (57) and in cultured rat hepatocytes (60). Circulating levels of IGF-I parallels the IGF-I mRNA abundance in liver in hypophysectomized (34,35), diabetic (61,62) and fasted rats (63). The developmental pattern of IGF-I gene expression in liver also parallels circulating levels of IGF-I. The above data support the concept that the liver is the major source of circulating IGF-I. D'Ercole et al (64) measured human serum and tissue concentration of IGF-I after 9 to 19 weeks of gestation. The highest concentration of IGF-I was found in lung and intestine. All the other tissues tested had significant concentrations of IGF-I, which could not be attributed to blood concentration. Fetal liver had the lowest concentration of IGF-

I of any tissue studied. These results support the view that IGF-I is produced in many fetal tissues and may have important roles in fetal growth and development. Multiple fetal tissues were also found to release IGF-I into the medium (65). Many tissues of rat other than liver contain IGF-I in excess of that attributed to the content of blood (66) and express IGF-I gene (50). A wide variety of cells also produce IGFs in cultures and express IGF genes (67-69), thus establishing their local biosynthesis and paracrine role.

(i) **Effect of growth hormone (GH) on local production of IGF-I**

GH has been established as a primary regulator of IGF-I gene expression in adult, not only in liver but also in many extrahepatic tissues, such as heart, lung, kidney and prancreas (50,51,70). GH stimulation of IGF-I gene expression and IGF-I protein synthesis is observed in heart and lung of adult hypophysectomized rat treated with GH. Similarly GH induction of skeletal growth is mediated by stimulation of local IGF-I production (71). GH has also been found to stimulate IGF-I production and IGF-I gene expression in cultured cells (68,69).

(ii) **Effect of trophic hormones on paracrine IGF-I biosynthesis**

Many trophic hormones appear to be able to regulate prarcrine IGF biosynthesis. Growth factors PDGF and FGF could stimulate IGF-I production from human fibroblasts in vitro (72). Trophic hormones stimulate the paracrine biosynthesis of IGF-I from their target organs. ACTH stimulates IGF-I

production from adrenal cells (73), TSH from thyroid follicular cells (67), LH from sertoli cells (74) and FSH from granulosa cells (75). Thus the growth promoting effect of many hormones is mediated via stimulation of local IGF-I production.

In the postnatal animal, there is the possibility of an interplay of both autocrine/paracrine actions and endocrine actions of IGF-I. Which mode of action is more important may vary from tissue to tissue and may depend on the animal's stage of development. Autocrine/paracrine actions are probably important in tissues with high local IGF-I gene expression and IGF-I tissue concentrations such as lung, kidney and ovarian granulosa cells, and where anatomical barriers limit the entry of IGFs from the circulation as in the testis and central nervous system. Autrocrine and paracrine actions may have a particularly important role in hypertrophy and repair (76,77) (this is discussed in the section on "Regulation").

E. **SECRETION**

Most polypeptide hormones are synthesized in endocrine cells and stored in form of granules. The content of these granules is rapidly secreted into the blood stream when required and therefore, the hormone level in plasma may change rapidly over a wide range. This is entirely different in the case of the IGFs. The concentration of IGFs does not undergo diurnal variation and is very stable in any given subject under constant nutritional and endocrine conditions. There is little

preformed or stored IGF or IGF precursor molecules in livers. IGF is synthesized rapidly and is readily secreted (59).

F. REGULATION OF IGF PRODUCTION AND IGF GENE EXPRESSION

The study of IGF gene expression in rat, mouse and human, have revealed multiple transcripts for IGF-I and IGF-II mRNAs in a wide variety of tissues and cells (22,28,29,34,35,50,78,80). IGF-I and IGF-II mRNA expression in fetal tissues has also been shown by Han et al (81) by in situ hybridization histochemistry. Most tissues synthesize both growth factors at some time during pre or postnatal development, the only exception being fetal neurons in culture, which produce only IGF-I (82). The regulation of IGF-I and IGF-II production in serum is usually measued by RIA, and their mRNA expression has been studied by quantitative determination of their respective mRNAs mainly using Northern blotting. Changes in mRNA levels could be due to the changes in transcription rates of the gene or due to change in mRNA stability. IGF-I and IGF-II production and their gene regulation are under the control of various factors which will be considered under the separate headings.

(a) IN VIVO

(i) Developmental

In serum

In human, IGF-I levels are low prenataly and at birth. This could be because of the lack of mature GH receptor, GH

fails to stimulate fetal IGF-I production (83) and does not elicit "growth response" during early development (84). IGF-I levels rise during childhood to high levels during puberty, after which they decline with increasing age (85). Serum IGF-I concentration in girls tend to be higher than in boys consistent with their more rapid maturation.

During puberty, there is 2- to 3- fold rise in IGF-I concentrations in serum with higher values in girls than boys. The rise in IGF-I correlated better with Tanner pubertal stage (86) and sex hormone concentrations (87). There is a good correlation between IGF-I and linear growth velocity until peak velocity is reached. Thereafter, IGF-I concentrations continue to rise as growth velocity subsides as a result of the terminal maturation of the cartilage growth plates. There is a gradual decline of the higher pubertal IGF-I concentrations, and average adult levels are reached early in the third decade.

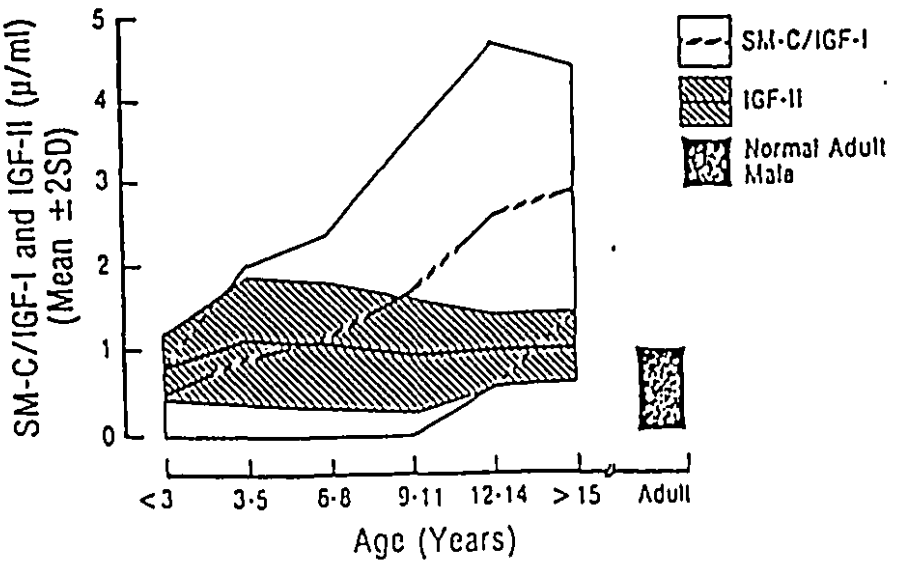
Serum levels of IGF-I and of IGF-II during infancy and childhood are represented in figure 4 (88).

A similar pattern for IGF-I has been found in rats where IGF-I levels in serum begin to rise at the time when growth becomes GH dependent (89).

The IGF-II pattern differs between human and rats. In rats, the serum levels of IGF-II are high prenatally and decline rapidly after birth (90), whereas in humans, levels are low prenatally and there is a rise during the first postnatal year (91). After the first years of life, the IGF-II levels in

Figure 4

Serum IGF-I and IGF-II levels during infancy and childhood



Schematic representation of serum SM-C/IGF-I and IGF-II levels by RIA during infancy and childhood. The adult reference has an arbitrary designation of 1 unit/ml. In this assay, 1 unit of IGF-I was equivalent to 180 ng, and 1 unit of IGF-II was equivalent to 540 ng. Taken from reference 88.

healthy subjects remain four times higher than those of IGF-I throughout life (92).

IGF gene expression

In rat IGF-I mRNA increases nearly 9 fold between days 11 and 13 of gestation. This increase corresponds to a time when organogenesis is occuring and contrasts with IGF-II mRNA levels which remain high but constant over the same developmental period (80). This study and others (52,93) demonstrate that the ontogeny of IGF-I gene expression precedes that of GH and reinforces the idea that IGF-I as well as IGF-II may play critical roles in fetal differentiation or in other developmental processes. In rat and humans, the abundance of IGF-II mRNA is highest during fetal development, and it declines in the postnatal period (29,50,52,78,79). In the rat, all IGF-II mRNA species exhibit co-ordinate development expression (79,94). In some human tissues such as liver (95), there appears to be a developmentally related switch in promoter usage; while in other organs, such as kidney (19), no change occurs in mRNA or the promoter which is activated.

In general, during fetal life the content of IGF-II mRNA in any given organ or tissue remains constant, although in rat brain, mRNA levels decline after embryonic day 14 (29,82). Of all rat tissues, the brain is exceptional in that brain IGF-II mRNA content remains high into adult life. Most of the brain IGF-II mRNA appears to be synthesized by the cells of the choroid plexus and by the leptomeninges, although some is

produced by glia cells as well (82,96).

(ii) **Tissue specific**

IGF gene expression

Tissue specific factors are involved in the regulation of IGF-I gene expression. IGF-I mRNA levels vary by nearly 2 orders of magnitude in the adult rat tissues, with liver having the higest concentration of any rodent tissue (50,70, 52,67,82). Within an organ, there may be cell type or regional differences in IGF-I mRNA abundance. Thus in the central nervous system IGF-I mRNA is distributed asymmetrically, with the cervical-thoracic spinal cord and olfactory bulb containing 8-10 times as much as the whole brain (82). In the rat kidney, nearly 10 times as much IGF-I mRNA is found in the cells of the collecting duct as in other parts of the nephron, and IGF-I protein can be detected by immunocytochemistry only in the principal cells of the collecting duct (97). These observations indicate that IGF-I mRNA is not produced exclusively by connective tissue cells of mesenchymal origin (81) but also by parenchyma. Tissue specific factors also play a role in IGF-II gene expression. Human and rat fetal liver, skeletal muscle, and skin contain high levels of IGF-II mRNA whereas the hypothalamus, cerebral cortex, brain stem and thymus contain relatively low levels (28,78,93). In adult rat brain IGF-II mRNA levels are maintained and far exceed those of other tissues (29,79,82,93). The choroid plexus in the adult rat

brain is a primary site of synthesis of IGF-II, a probable source of IGF-II in cerebrospinal fluid and a potential source of IGF-II for actions on target cells within the adult brain (98).

IGF-I and IGF-II mRNA levels in fetal and adult, rat and human tissues are summarized in Table 1 (99). These results clearly indicate the developmental and tissue specific regulation of IGF-I and IGF-II gene expression in rat and human.

(iii) **Nutritional**

In man

Nutrition has been suggested to play a primary role in the regulation of IGF production throughout life (100). During acute fasting in normal adults, the decline of IGF-I levels is more pronounced than IGF-II levels. The IGF-I levels correlate to the change in nitrogen balance and adequate nutrition is required for adequate effect on IGF-I generation (101). Only refeeding elevates the IGF-I levels after fasting, and both protein and energy are important determinants of IGF-I, with energy possibly being a somewhat more important determinant than protein (102). In GH deficient patients, IGF-I and IGF-II responses to GH were inhibited during fasting (103). These observation suggest a close correlation of IGFs to catabolic responses in states of undernutrition and starvation.

Table 1

Expression of IGF-I and IGF-II mRNAs in rat and human tissues

	IGF-I		IGF-II	
	Human	Rat	Human	Rat
Expression in fetus				
Liver	+	+	+++++	+++++
Brain	+	+	+/-	++++
Extra-hepatic	+	+	+++++	+++++
Expression in adult				
Liver	+++++	+++++	+	+
Brain	ND	+	ND	+++
Extra-hepatic	+	+	ND	-/+
GH dependence				
Liver	ND	++++	ND	-
Brain	ND	++	ND	+
Extra-hepatic	ND	+	ND	-/+

ND = not done; - = not detected or no detectable regulation

IGF-I and IGF-II mRNA levels in fetal and adult human and rat tissues. Tissue specific and developmental specific expression of IGF-I and IGF-II mRNAs are denoted in the above table. The abundance of IGF-I and IGF-II mRNAs are denoted by the number of pluses (+). Adult human and rat liver contain more IGF-I mRNA than does the fetal liver tissue, whereas fetal human and rat liver contain more IGF-II than adult liver tissue. Fetal brain also contains more IGF-II than adult brain. Taken from reference 99.

In the rat

The nutrition dependency of IGF-I levels has been confirmed in rats (104). In neonatal rats, both IGF-I and IGF-II levels seem to be dependent on caloric intake (104). In rats, fasting results in a decrease in cartilage growth and a decline in immunoreactive IGF-I (105). Although GH levels are low in fasting rats (106), administration of exogenous GH is ineffective in restoring somatomedin levels (105) suggesting a state of GH resistance.

During fasting, hepatic IGF-I mRNA transcripts in the rat have been shown to decrease progressively. Refeeding caused a marked rise in IGF-I mRNA abundance (63,107). Serum IGF-I concentrations paralleled the levels of hepatic IGF-I mRNA during periods of both fasting and refeeding (63). Hepatic IGF-I mRNA levels were also found to decrease in rats fed a protein-free diet for 1 week. There was also a decrease in immunoreactive IGF-I in the plasma of these rats. No significant difference was found in the rate of hepatic IGF-I gene transcription in these rats (63,107), suggesting that the decrease in hepatic IGF-I mRNA in these animals was partly regulated at a posttranscriptional level. Nutritional factors have no effect on regulation of IGF-II mRNA levels whereas they regulate IGF-I mRNA levels possibly by affecting GH receptor mRNA levels in a similar manner. The nutritional status of the rat is an important determinant in the regulation of the growth-promoting system (63).

(iv) **Hormonal**

Growth hormone effects

Growth hormone (GH) has been shown to be an important regulator of IGF-I production. IGF-I concentration is low in hypophysectomized rats and reaches a normal level after GH therapy (66). However, the response to GH is developmentally dependent, since, presumably because of the lack of mature GH receptors, GH fails to stimulate fetal IGF-I production (108).

During pregnancy

The changes which occur during pregnancy have been described by a number of investigators (109-111). The rise in maternal serum IGF-I that occurs during the last trimester of pregnancy is not pituitary dependent (110,112). It has been suggested that placental lactogen (PL) in fetal placental unit is responsible for this induction of maternal IGF-I during pregnancy. It has been recently demonstrated that a GH-like molecule progressively rose and reached a high level at term (113). This could be a variant form of hGH (hGH-V). hGH-V gene is expressed in human placenta (114). At this point it is not known whether stimulation of maternal serum IGF-I level is due to increased level of serum PL or serum hGH-V in late pregnancy.

On tissue levels of IGFs

Tissue levels of IGF-I and IGF-II are much lower than serum levels (115). The highest concentrations of IGF-I have been

detected in kidney, liver, lung and testis (115,116) and these tissues also express IGF-I mRNAs (50,51,70). After hypophysectomy, IGF-I concentrations fell to very low levels in liver and to a lesser extent in other tissues. After the administration of ovine GH to hypophysectomized rats, tissue extractable IGF-I increased earlier than serum IGF-I. This argues for an important role of autocrine/paracrine actions of IGF-I in the early phase of the response to GH.

On IGF-I mRNA expression

The primary regulator of IGF-I mRNA level is growth hormone (GH). GH enhances IGF-I mRNA transcription (70) and increases IGF-I mRNA abundance in most rodent tissues (34,35,51,70,117). The effect is rapid with peak levels of IGF-I mRNA being achieved within 3-9 h after a single GH injection in both GH-deficient and normal animals (35,70,118) also in primary culture (60) and in established cell lines (118).

All species of IGF-I mRNA increase after GH administration, although the magnitude of change varies with the tissue and the subclass of mRNA. It has been shown that the three rat IGF-I mRNAs with different 5'-ends increase in the liver after GH treatment, while in the kidney and lung the abundance of only one mRNA subtype is altered (117).

Chronic GH excess leads to an increase in IGF-II mRNA in rat skeletal and cardiac muscle but not in liver (119). The effect of GH on IGF-II mRNA levels in hypophysectomized rat brain (99) is controversial as some studies have described stimulation but

other studies have demonstrated an inconstant response (51).

On Growth and IGFs in humans

IGF-I activities are decreased in hypopituitary dwarfs and are increased with GH administration. IGF-II levels also increase after GH administration (119). There are a number of conditions in which a discrepancy occurs between estimates of GH secretion and circulating IGF-I values.

(a) In acromegaly, IGF-I values are invariably elevated despite low basal GH concentrations (120).

(b) Laron-type dwarfism is characterized by elevated GH levels and very low levels of IGF-I and IGF-II (121). These patients have an inability to secrete and/or synthesize IGFs and present a resistance to endogenous and exogenous GH.

(c) A combination of normal serum GH and low IGF-I values with growth failure of the hypopituitary type has suggested a biologically inactive GH in such a case (122). Some of these patients have been responsive to exogenous administered GH.

Effect of other hormones

Estrogen

In man

Estrogen's effect depends on its dosage. High doses of estrogen cause a decrease in IGF-I levels and rise in IGF binding proteins during the early phase of therapy (123). Low doses of estrogens enhance IGF-I concentrations when given to

normal prepubertal children (87). This rise is associated with an increment in GH secretion (87). IGF-II values remained unchanged in these patients. Clemmons et al (124) have reported that pharmacological doses of estrogens reduce serum IGF-I levels in patients with acromegaly. This appears to be the result of a direct inhibitory effect of estrogen on somatomedin production, since estrogen had no effect on the growth hormone levels in these patients (124). These results suggest a biphasic effect of sex steroids on IGF-I production. Low levels of sex steroid stimulating IGF-I production by stimulating GH secretion and high levels of sex steroid directly suppressing IGF-I production. The slow decline in IGF-I level which is observed with advancing age appears to be the result of a decrease in pituitary GH secretion, since administration of exogenous GH to adults causes a substantial increase in serum IGF-I levels (125). It has been suggested that estrogens may also be responsible for the fall in the IGF-I concentration which occurs during the 3rd decade (124). There is no evidence that estrogens directly stimulate IGF-I production.

In the rat

Estrogens rapidly increase IGF-I mRNA content in the uterus of ovariectomized, hypophysectomized rats as well in pituitary intact, ovariectomized rat, yet inhibit GH stimulated hepatic IGF-I gene expression (126). Estradiol has no effect on hepatic IGF-II mRNA levels of 1 day old rat (127). Estrogen rather than GH has been shown to be the most important regulator of uterine

IGF-I expression (126) and presumably of IGF-I production in uterus.

Prolactin

In man

Serum prolactin and IGF-I levels are slightly elevated after surgery in patients with pituitary tumors, in spite of GH deficiency (128). This suggests that prolactin may be stimulating serum IGF-I levels in such patients.

In the rat

In adult rats, administration of ovine prolactin (oPRL) resulted in a 15 fold increase in hepatic IGF-I mRNA abundance 12 h after administration. Serum IGF-I also increases 2.5 fold (129). oPRL, like human GH has both somatogenic and lactogenic activity in the rat (130) and it has been suggested that the effect of oPRL on IGF-I production may be due to its somatogenic activity (129).

Glucocorticoids

In man

IGF-I values are normal in Cushing's Syndrome or in children receiving glucocorticoid therapy (131), despite significant growth ratardation. Glucocorticoids are particularly potent in inhibiting growth, and children who receive exogenous glucocorticoids often demonstrate poor growth. Glucocorticoids

have been shown to inhibit sulfate incorporation into cartilage both in vivo and in vitro (132).

Dexamethasone

In the rat

Dexamethasone reduces hepatic IGF-I and IGF-II mRNA abundance in the rat (127,133), and inhibits growth hormone induction of hepatic IGF-I mRNA in hypophysectomized rats (133). Hepatic IGF-II mRNA levels remained low after withdrawal of dexamethasone (133). Dexamethasone also reduces GH stimulated serum IGF-I levels in hypophysectomized rat (133).

Thyroid hormone

Studies in humans and animals have shown that thyroid hormone stimulates growth minimally if at all, in the absence of GH (134,135). On the other hand GH stimulates growth significantly better in the presence than in the absence of thyroid hormone (136). The precise mechanisms by which thyroid hormone regulates growth and development remain unclear. Some of the growth promoting effect of thyroid hormone appears to be the result of its ability to interact with the GH IGF-I axis. Both basal and stimulated GH levels are diminished in the hypothyroid state (137) and pituitary GH content is diminished markedly in hypothyroid animals (138). These observations have led to intensive investigation of the transcription regulation of the GH gene by thyroid hormone (139). The number of GH receptors in the liver has also been shown to change with

thyroid status of the animal (140); increasing in the hyperthyroid state and decreasing in the hypothyroid condition. This effect of thyroid hormone is a direct effect on GH receptors. The effects of thyroid hormone, in the presence and absence of GH, on hepatic IGF-I mRNA levels and IGF-I peptide production have been investigated in hypophysectomized rats (141).

IGF-I concentrations are low in hypothyroid patients and increase during thyroxine therapy (142). Because IGF-I responses to administered GH were not impaired, it is likely that these changes are largely due to the reduced secretion of GH observed in hypothyroid patients (134). Thyroid hormone alone has relatively little effect on circulating IGF-I levels and on the stimulation of hepatic IGF-I mRNA levels in hypophysectomized rats, but it does potentiate the effects of GH on hepatic IGF-I mRNA abundance and circulating levels of IGF-I (141).

Testosterone

Testosterone stimulates IGF-I at low doses and after short term administration (143). This effect is associated with an increment of GH levels. This response does not occur in hypopituitary patients (144). There is no evidence for a direct effect of testosterone on IGF-I production.

Estrogen and testosterone under physiological conditions influence IGF-I levels via an increase in GH secretion rather

than an increase in responsiveness to GH (145).

LH and FSH

IGF-I mRNA levels are stimulated 7 fold by LH and 6 fold by FSH in the testis of immature, hypophysectomized rats (22 days old). No significant change in plasma IGF-I concentration was observed after LH or FSH administration (146). LH and FSH had no effect on IGF-II mRNA levels in the hypophysectomized rat testis (146). LH and FSH may stimulate the local production of gonadal IGF-I but appear to have no effect on circulating IGF-I levels in the rat. GH stimulates both IGF-I mRNA levels in rat testis and circulating levels of IGF-I in rat serum. This effect of GH on testis IGF-I mRNA may be indirect as no GH receptors have detected in rat testis.

Somatostatin

The long acting somatostatin analog octreotide (SMS), has a direct inhibitory effect on human growth hormone (hGH) release by cultured pituitary tumor cells from acromegalic patients (147) and has proven to be an effective treatment for acromegaly (148). Recent studies indicated that in several SMS-treated acromegalic patients, serum IGF-I decreased relatively more than hGH perhaps indicating inhibition of SMS on GH action to augment IGF-I production (148). We investigated the inhibitory effect of octreotide on IGF-I production and hepatic IGF-I gene expression in hypophysectomized rats and in cultured rat hepatocytes (149). Octreotide had no effect on hepatic IGF-

I mRNA levels or circulating levels of IGF-I in hypophysectomized rats; but it inhibited the effect of GH on the stimulation of hepatic IGF-I mRNA levels and circulating levels of IGF-I (149). This inhibitory effect of octreotide on GH stimulation of IGF-I production could be due to a direct effect on liver to inhibit the increase in IGF-I mRNA levels (our observations in cultured rat hepatocytes) and partly due to an inhibitory effect on serum glucagon levels in hypophysectomized rats. We have previously shown that glucagon synergizes with GH to augment hepatic IGF-I mRNA levels and IGF-I peptide production in cultured rat hepatocytes (60).

In Diabetes

In man

It has long been known that insulin dependent diabetes mellitus (IDDM) is associated with growth retardation (150). Administration of insulin is associated with an increase in the growth rate of diabetic children (151). Serum IGF-I levels appear to be decreased in uncontrolled IDDM and this may occur in spite of increased GH levels (151). The levels of IGF-I are clearly related to the degree of control of diabetes (152). Intensive insulin therapy is necessary to maintain normal IGF-I levels (153). IGF-I and IGF-II concentration are not increased in children with hyperinsulinism (154).

In the rat

IGF-I levels in rats with streptozotocin induced diabetes (STZ-D) have been found to be reduced (155). In contrast to humans, GH levels are decreased (156) and GH receptors have been reported to be low (157). The inability of GH to generate an increase in circulating IGF-I levels in diabetes has previously been reported in both humans (158) and animals (159). Liver GH binding sites are depleted shortly after induction of severe diabetes (160).

In streptozotocin induced diabetic rats hepatic IGF-I mRNA levels are reduced and are restored by insulin therapy (61,62). This effect is not due to GH deficiency in diabetic rats, as GH administration is ineffective in restoring serum IGF-I levels in these animals. Insulin therapy was found to restore the basal and GH-induced IGF-I mRNA level in liver and also in other tissues such as kidney and lung (62). Decreased expression of the IGF-I gene in diabetes may be an important determinant of the growth retardation observed in this disorder (62).

Placental lactogen

In man

Human placental lactogen (hPL) levels correlate with IGF-I values during late pregnancy in normal women and in GH deficient patients (111). hPL appears to regulate serum levels of IGF-I in pregnancy rather than GH. During pregnancy IGF-II levels do not change significantly.

(v) **In pathophysiological conditions**

IGF levels have been found to vary in different pathophysiological conditions. In liver insufficiency, IGF-I and IGF-II levels are depressed (161). It is not clear to what extent IGF values correlate with minimal early changes in liver dysfunction. In thalassemia major, low IGF-I values have been observed inspite of normal or elevated GH concentrations and there is a lack of responsiveness to GH administration (162). In children with chronic renal failure, IGF-I levels appear to be low or normal for age whereas IGF-II levels are elevated (163).

(vi) **In tissue hypertrophy**

Renal IGF-I gene expression is enhanced in compensatory renal hypertrophy occuring after unilateral nephrectomy (164). IGF-I and IGF-II mRNA abundance are also increased in skeletal muscles during muscle hypertrophy (165). This induction is independent of GH and of other pituitary hormones since it occurs in hyopophysectomized rats. These studies indicate that enhancement of paracrine IGF biosynthesis occurs during the repair of tissue damage.

(vii) **In human tumors**

Work by many investigators have established that a wide range of tumors manifest increased IGF-II gene expression (Table 2) (166). These tumors are also found to contain IGF-II

Table 2

IGF-I and IGF-II peptide production and mRNA expression in human tumors

Tumors	IGF-I		IGF-II	
	mRNA	peptide	mRNA	peptide
Sarcoma				
Fibro-			+	+
Hemangiopericyte-			+	+
Leiomyo-			+	+
Lipo-			+	
Mesothelial-			+	+
Rhabdomyo-			+	+
Ewing's	+			
Neural				
Pheochromocytoma			+	+
Neuroblastoma			+	+
Neuroepitheloma	+			
Carcinomas				
Breast	+	+		
Colon	+	+		
Hepatoma			+	
Wilm's			+	+

Table 2

Various human tumor tissues contain IGF-I and IGF-II peptides and express their mRNAs. Taken from reference 166.

peptides. Many of the tumors which have increased levels of IGF-II mRNAs also contain IGF-I mRNAs, but the amount of IGF-I mRNA present represents a small increase compared to the corresponding normal tissue. Haselbacher et al (167) found that pheochromocytomas contained greatly increased concentrations of IGF-II, most of which was present as a 10 kilodalton (kDa) species suggestive of a partially processed proIGF-II. Most of the IGF-II present in fibrosarcoma (originally identified as a leiomyosarcoma) is the large mol wt (big) IGF-II (168).

The intense research in this field has established that IGFs, particularly IGF-II, are synthesized by many tumors, but that processing of proIGF-II is frequently incomplete. Many of the same tumors contain IGF-I receptors and mitogenesis of several cell lines is stimulated by added IGFs. The ability of several tumor cell lines to proliferate in serum free medium is dependent on the autocrine/paracrine action of IGFs. Certain tumors produce sufficient IGF-II to suppress GH and IGF-I secretion and induce hypoglycemia (168). The role of IGFs in tumor growth or progression is not known.

(b) **IN VITRO**

(i) **In rat liver perfusate**

The regulation of somatomedin production in liver perfusate has been studied by several investigators. Somatomedin levels decline in diabetic (169) and hypophysectomized (170) rats and are partly restored by insulin and GH therapy respectively (169,170). IGF-I levels are also reduced in starved rats

(169,171) and are normalized on refeeding. This is not due to GH deficiency as GH fails to restore IGF-I levels to normal in fasted rats. GH, oPRL, insulin and cortisol appear to stimulate somatomedin production in perfused liver from normal (170,172) and hypophysectomized rats (170).

(ii) **In cultured rat hepatocytes**

Various hormones and agents have been shown to stimulate or inhibit IGF-I mRNA levels or IGF-I peptide production in adult rat hepatocytes in culture. (Table 3).

These cells have been shown to express IGF-I mRNA and secrete IGF-I peptide in to their culture medium. The effect of GH on IGF-I production in hepatocytes has been studied by several investigators (Table 3). Johnson et al (173) showed that GH and insulin individually stimulated IGF-I mRNA levels and IGF-I peptide production, but no additive effect on stimulation of IGF-I mRNA levels and IGF-I peptide production was observed when insulin and GH were combined. Boni-Schnetzler et al (174) showed a stimulatory effect of each of GH and insulin and an additive effect of GH plus insulin on IGF-I mRNA levels in hepatocytes. The culture medium used in one study contained T_3, hydrocortisone and insulin (173) and in the other contained dexamethasone and insulin (174). And in both cases, the experiments were carried out after 24 h of initiating the culture.

We observed that maintaining hepatocyte cultures in a serum-

Table 3

Regulation of IGF-I mRNA levels and IGF-I peptide production in normal adult rat hepatocytes.

Effector	Response		Ref
	IGF-I mRNA	IGF-I peptide	
GH	↑	↑	60,173,174,176,177
Ins.	↑	↑	173,174,177-179
Ins.	↓		175
PMA	→		177
PMA	↑		178,175
cAMP	↑		177,175
Glucagon(G)	↑	↑	60,177
Dex.	→		175
T_3	→		175,176
oPRL	→		175
Tryptophan	↑	↑	180
Cyx.		↓	178
Act. D		↓	178

Table 3

Adult rat hepatocytes secrete IGF-I peptide in their culture medium and express IGF-I mRNA. IGF-I mRNA levels and peptide production are regulated by various hormones and agents which stimulate (↑), inhibit (↓) or have no effect (→) on IGF-I production in hepatocytes.

free medium for 72 h was essential to obtain a maximum response to bGH on IGF-I mRNA stimulation. We also observed that insulin inhibited IGF-I mRNA levels in hepatocytes in the presence of bGH, glucagon or bGH plus glucagon (175). These results indicated that hepatocytes maintained in culture for 72 h responded reliably to the hormone of interest compared to hepatocytes maintained for 24 h in culture with the additional hormonal supplements in the medium. Tollet et al (176) also showed no effect of insulin on IGF-I mRNA levels in hepatocytes. They maintained the hepatocytes in culture for 66 h prior to treatment. Our studies indicated that glucagon and bGH individually stimulated IGF-I mRNA levels 1.8 to 2.0 fold and 2.0 to 2.5 fold respectively but when combined, produced a synergistic effect and stimulated IGF-I mRNA levels 10 to 12 fold (60). The stimulatory effect of glucagon and bGH on IGF-I peptide production was also observed in hepatocytes (60). Tollet et al (177), recently reported the stimulatory effect of glucagon on IGF-I mRNA levels in hepatocytes but no synergistic effect was observed when glucagon and bGH were combined (177).

We have also reported an effect of glucagon on GH stimulation of hepatic IGF-I mRNA levels and serum IGF-I levels in hypophysectomized rats in vivo (149). Octreotide, a somatostatin analog, inhibited the GH stimulatory effect on hepatic IGF-I mRNA and serum IGF-I levels in hypophysectomized rats. This inhibitory effect of octreotide was found to be partly due to low serum glucagon levels when GH and octreotide were administered in hypophysectomized rats and partly due to a

direct effect of octreotide on the inhibition of the GH effect on IGF-I mRNA levels in hepatocytes (149).

Like glucagon, IBMX and $(Bu)_2$cAMP stimulated IGF-I mRNA levels 1.8 to 2.0 fold but produced a synergistic effect and stimulated IGF-I mRNA levels 10 to 12 fold when added in combination with bGH. PMA when added in combination with bGH, had no synergistic effect on IGF-I mRNA stimulation in hepatocytes. These data indicated that glucagon when added in combination with bGH acts via the cAMP pathway rather then via the PKC pathway to stimulate IGF-I mRNA levels.

Trophic hormones have been shown to stimulate IGF-I production in various cell types . In all instances the stimulation of cAMP production occurs concomitantly. ACTH stimulates IGF-I production in bovine adrenal cells (73), FSH in granulosa cells (75), LH in sertoli cells (74), and TSH in thyroid follicular cells (67). Glucagon has been implicated as a trophic factor for regenerating liver (116).

The synergistic effect of bGH plus glucagon on IGF-I mRNA stimulation was markedly inhibited in (a) PKC depleted cells; (b) in the presence of inhibitors of protein kinase C; and (c) in the presence of cycloheximide (175). These results indicate that bGH and glucagon act via protein kinase A (PKA) and PKC stimulation and may also require synthesis of one or more new protein(s) to synergize in the stimulation of IGF-I levels in hepatocytes. No synergistic effect on IGF-I mRNA stimulation in hepatocytes was observed by combining PMA and $(Bu)_2$cAMP. These

results indicate that activation of PKA and PKC is necessary but not sufficient to synergistically stimulate IGF-I mRNA levels in hepatocytes.

Glucagon and $(Bu)_2$cAMP act in part to stabilize IGF-I mRNA at a posttranscriptional level. Dexamethasone, T_3 and oPRL had no effect on IGF-I mRNA stimulation whereas EGF and insulin inhibited IGF-I mRNA levels in the presence of bGH or glucagon. These data showed that GH and glucagon are the major regulatory hormones of hepatic IGF-I mRNA levels. The stimulatory effect of insulin observed by others in vivo in the rat (61) could be an indirect effect on hepatic IGF-I mRNA levels.

The inhibitory effect of dexamethasone on hepatic IGF-I mRNA levels observed in vivo (142) is not seen in vitro in hepatocytes (175). oPRL has also been shown to stimulate hepatic IGF-I mRNA levels in vivo in the rat (131), whereas no stimulatory effect on IGF-I mRNA levels is observed in hepatocytes in vitro (175). The results obtained in vivo in the rat appear to differ from those obtained in vitro in cultured hepatocytes. The in vivo effect could be indirect and result from interactions of various hormones, whereas in hepatocyte cultures the direct effect of various hormones under controlled conditions can be evaluated.

(iii) **In various cell types in culture**

GH has been shown to stimulate IGF-I mRNA levels and IGF-I peptide production in various cell types as shown in Table 4.

Table 4

Regulation of IGF mRNA levels and their peptide production in various cell types

Cells	Effector	IGF-I mRNA	IGF-I peptide	IGF-II mRNA	IGF-II peptide	Ref
Porcine preadipocyte cultures	GH	↑	↑			68
Fetal porcine preadipocyte cultures	GH	↑	↑			68
Porcine sertoli cells	LH		↑			74
Porcine granulosa cells	GH		↑			181
	Cyx		↓			
	LH		↑			75
	FSH		↑			
	Estrogen		↑			
	cAMP		↑			
	EGF		↑			182
	TGF-α		↑			
Rat adipose Ob1771 cells	GH	↑				119
Rat pituitary GH_3 cells	GH	↑				69
	T_3	↑				
Rat skin fibroblast	FCS	↓				183
	Ins.	↑				
	IGF-I	↑				

Table 4 (CONT'D)

Cells	Effector	IGF-I mRNA	IGF-I peptide	IGF-II mRNA	IGF-II peptide	Ref
Rat neuronal and glial cells	Dex.	↓				184
Rat ovarian theca-interstial cells	estrogen				↓	185
Rat embryo fibroblasts	PL				↑	186
Adult rat fibroblasts	GH		↑		→	186
	PL		↑		→	
Bovine adrenal cells	FGF		↑			73
	A-II		↑			
	ACTH		↑			
	GH		→			
Human thyroid follicular cells	TSH		↑			67
	GH		↑			
Fetal rat osteoblast	estradiol	↑	↑			187
	PGE_2	↑	↑			188
	PTH	↑	↑			189
	cAMP	↑	↑			190
Rabbit chondrocytes	Ins		↑		↑	
	IGF-I				↑	191
	IGF-II		↑			
	bFGF		↑			192

TAble 4

Various cell types secrete IGF-I and IGF-II peptides in their culture media and express their mRNAs. Several hormones, growth factors and agents either stimulate (↑), inhibit (↓) or have no effect (→) on IGF-I or IGF-II peptide production or their mRNAs expression in these cells.

GH has no effect on IGF-I production in rat embryo fibroblasts but instead placental lactogen (PL) stimulates IGF-II production from these cells (186). But in adult rat fibroblasts, there is a switch and GH as well as PL stimulate IGF-I production whereas they have no effect on IGF-II production (186). These data support the notion that IGF-II is a fetal growth factor whereas IGF-I is important in postnatal growth.

Trophic hormones appear to stimulate IGF-I production from their target cells and also cAMP has been shown to produce similar effect on these cells. LH stimulates IGF-I production in Sertoli cells (74), whereas LH, FSH, estrogen and cAMP do so in granulosa cells (76), ACTH in bovine adrenal cells (73), TSH in thyroid cells (67), and PTH and cAMP from fetal rat osteoblast (189,190). Trophic hormones appear to stimulate production of IGF-I by stimulation of cAMP levels. Fetal calf serum has been shown to contain an inhibitory factor which inhibits IGF-I mRNA levels in rat fibroblasts (183). Insulin has been shown to stimulate IGF-I production in several cell lines. IGF-I, IGF-II and fibroblast growth factor (FGF) also appear to stimulate IGF-I production in various cell types. The data presented in Table 4 summarize autocrine/paracrine regulation of IGF-I and IGF-II production in various cells.

(iv) **In human cell lines in culture**

Various hormones or factors regulating IGF-II mRNA levels in human cells in culture are listed in Table 5. Trophic hormones

Table 5

Regulation of IGF-II mRNA levels in human cell cultures

Cells	Effector	IGF-II mRNA	Ref
Human fetal adrenal cells	ACTH	↑	193
	IGF-I	↓	
	IGF-II	↓	
	cAMP	↑	
Human ovarian granulosa cells	cAMP	↑	194
	hCG	↑	194,195
	FSH	↑	194,195
	LH	↑	195
Human fetal testicular cells	FSH	→	193
	hCG	→	
	cAMP	→	
Human placental cells	cAMP	↑	194
	hCG	→	
	hGH	→	

Table 5

Several human cell types express IGF-II mRNA in culture. IGF-II mRNA levels are either stimulated (↑), inhibited (↓) or have no effect (→) when treated with various hormones or agents.

appear to stimulate IGF-II mRNA levels from their target cells and their effects appear to be mediated via elevation of cAMP levels. ACTH and cAMP stimulate IGF-II mRNA levels in human fetal adrenal cells (193), whereas FSH, LH and cAMP do so in human ovarian granulosa cells (194,195). It appears that trophic hormones are capable of stimulating IGF-I as well as IGF-II production in their target tissues. But IGF-II is mainly produced by fetal tissues or fetal cells in culture.

(v) **In human cancer cell lines**

IGFs may be autocrine growth factors for human cancer cell lines. Media conditioned by breast cancer cells contained an immunoreactive IGF-I related peptide (196). These breast cancer cells also express IGF-I mRNA. T-47D and MCF-7 cells were found to produce immunoreactive IGF-II and also contained IGF-II mRNA. T-47D is an estrogen responsive cell line and estradiol was found to increase IGF-II mRNA levels, whereas this increase was inhibited by antiestogen tamoxifen (197). There is only one report of expression of IGF-II mRNA in human lung cancer cell line NSCLC (198). These cells have been shown to secrete immunoreactive IGFs in their culture medium.

II. IGF BINDING PROTEINS

The insulin-like growth factors (IGF) I and II circulate in the plasma complexed to high affinity binding proteins (199-202). These binding proteins are specific to IGFs and do not bind insulin or proinsulin. There is no structural homology

between IGF binding proteins and type I and type II IGF receptors (203).

Currently six IGF-binding proteins have been identified and their cDNAs have been cloned from humans and rats (204-212). These binding proteins are designated according to the proposal adopted during a workshop on the IGF binding proteins held in Vancouver, B.C. Canada, June 17-19, 1989 (213) and in San Francisco, CA, USA on Jan 16, 1991 (214). Six binding proteins have been designated as IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6. The identification of binding proteins in body fluids and in the conditioned media from cultured cells has been greatly facilitated by the technique of ligand blotting (215).

A. TYPES

(a) IGF-Binding protein-1 (IGFBP-1)

The characterization of IGFBP-1 resulted from studies of at least four distinct proteins. (a) an IGFBP found in amniotic fluid (216,217), (b) and IGFBP in the conditioned media of a human hepatoma cell line HEP G_2 (218), (c) a placental protein, designated placental protein 12 (PP12) that was originally isolated from soluble extracts of term human placenta (219,220) and (d) a pregnancy-associated endometrial α_1-globulin (α_1-PEG) originally termed endometrial protein 12 (221,222), that is a major secreted protein of decidual stromal cells.

An acid-stable protein with an estimated molecular weight of

35-40,000 was first identified by Chochinov et al (216) and was further characterized by Drop et al (217). Using a polyclonal antibody generated against amniotic fluid binding protein (AFBP), AFBP was shown to be the same as "Small BP" found in fetal and newborn sera (223). Baxter et al (224) called this protein BP-28 because of its apparent molecular mass on SDS-PAGE under nonreducing conditions (M_r = 28,000 non reduced, M_r = 34,000 reduced).

IGFBP in media conditioned by the human hepatoma cell line HEP G_2 was isolated by Moses et al (218). This protein was found to have the same molecular mass as AFBP. Moreover the amino acid compositions of the two proteins were equivalent and N-terminal sequences were identical (225).

Human placental protein P12 (PP12) was first isolated from soluble extracts of human placenta (219). The molecular weight was in the range of 25,000 by utracentrifugation to 51,000 by SDS-PAGE. PP12 was demonstrated to be a decidual rather than a placental protein (226). The first 15 amino acids were subsequently demonstrated to be identical to the IGFBP isolated from amniotic fluid (220).

The pregnancy associated endometrial protein α_1-globulin (α_1-PEG) has molecular weight of 29-32,000. It was also known as endometrial protein 12 (221). It has been shown to be a minor secretory product during the menstrual cycle, but the major secretory protein of the decidua during pregnancy. It is similar to PP12 immunochemically (221) and binds IGF-I with high affinity (222). The N-terminal sequence of α_1-PEG reported

by Bell and Keyte (227) corresponds to primary structure predicted by the cDNA of PP12 (228) and the proteins are believed to identical. IGFBP-1 is present in human and rat sera (211,229) and is secreted by H4IIE rat hepatoma cells (230) and rat hepatocytes in culture (231).

(b) **IGF-binding protein-2 (IGFBP-2)**

IGFBP-2 was first identified by Moses et al (232) in rat BRL-3A cells. It was purified and shown to have M_r = 31,500 on SDS-PAGE. The first 30 amino acids from its N-terminus had limited identity with a sequence from AFBP/HEP G2 IGFBP-1 (234). A polyclonal antiserum against a purified BRL-3A IGFBP recognized M_r = 40,000 BP in neonatal rat serum, but failed to recognize M_r = 150,000 complex in adult rat serum (235) and AFBP. This suggested that IGFBP from BRL-3A cells was distinct from IGFBP-1 and IGFBP-3.

An IGFBP similar to the BRL-3A IGFBP was purified from conditioned media of bovine kidney (MDBK) cells (236). Like BRL-3A IGFBP, the bovine kidney (MDCK) BP had significantly higher affinity for IGF-II than for IGF-I. The estimated molecular weight was 40,000 on SDS-PAGE. The N-terminal amino acid sequence was identical to rat IGFBP-2.

Human IGFBP-2 is the major IGFBP present in human cerebrospinal fluid (CSF) (237). It did not react with antibodies generated against IGFBP-1. In the rat, IGFBP-2 has been shown to be the major IGFBP produced in the central nervous system (CNS) (238) and by fetal neuronal and astroglial

cells in culture (239). An IGFBP of similar size was subsequently identified in conditioned media of the human endometrial carcinoma cell line HEC 1A (240). This BP did not react with antibodies to IGFBP-1. Antibody generated against HEC 1A cell as well as antibody generated against rat IGFBP-2 reacted with BP from CSF. HEC 1A cells contain mRNA for IGFBP-2, suggesting that M_r = 31,000-34,000 protein is IGFBP-2. IGFBP-2 is present in human and rat serum (211,229) and has been shown to be a major fetal and neonatal binding protein in the rat (206).

(c) **IGF-binding protein-3 IGFBP-3**

Endogenous IGF activity in both human and rat plasma is predominantly associated with a protein of M_r = 150,000 complex. Two IGFBPs have been identified in human plasma (241), M_r = 150,000 and M_r = 40,000. The larger BP is usually saturated with IGFs whereas the smaller BP remains unsaturated. Acid treatment of the large BP results in irreversible dissociation of the complex and the release of bound IGF. M_r = 150,000 BP complex is composed of three subunits: IGF, a binding subunit and an acid labile subunit (242). IGF binds to GH dependent IGFBP of M_r = 50,000 which then binds to an acid labile subunit of M_r = 100,000 to form a M_r = 150,000 complex.

A GH dependent IGFBP purified from human plasma showed two bands, a major one at M_r = 53,000 and a minor band at M_r = 47,000 (243). Under reducing conditions these bands migrated at

43,000 and 40,000 respectively. The N-terminal sequences were the same for both BPs and significantly different from that of IGFBP-1. These BPs are the same as the M_r = 41,500 and 38,500 serum IGFBPs detected by Western ligand blotting (229). It has been shown that upon treatment with endoglycosidase F (Endo F), the larger bands are reduced in size to a single IGFBP of M_r = 29,000 (240). Thus the M_r = 53,000 and M_r = 47,000 proteins appear to be differentially glycosylated forms of the same IGFBP.

Similar proteins were also purified from adult rat serum with M_r = 56,000 and 50,000 non-reduced, and 48,000 and 44,000 reduced (224). N-terminal amino acids were identical in the rat and human GH dependent IGFBPs. IGFBP-3 is secreted by human (244) and bovine (245) fibroblasts, C_6 glial cells and B 104 neuroblastoma cells (246) in culture.

(d) **IGF-binding protein-4 (IGFBP-4)**

A low molecular weight IGFBP has been identified in both rat and human sera (211,229). A similar size BP has also been identified in conditioned media from human fibroblasts (247), human breast cancer cells (248) and seminal plasma (249). It has molecular weight of 24,000 to 30,000. Neuroblastoma cell line B 104 has been shown to produce glycosylated IGFBP-4 of Mol Wt. 28,000 and a nonglycosylated form of BP-4 of Mol. Wt. 24,000 (250).

(e) **IGF-binding protein-5 (IGFBP-5)**

A fifth IGFBP has been identified in human cerebrospinal fluid (251) and in human and rat sera (211,229). It has Mol Wt. of 34,000 by SDS-PAGE. It has ten times higher affinity for IGF-II than IGF-I. It appears to be a minor component of human CSF, unlike the most prevalent IGFBP-2. Human bone extract (252) contains IGFBP-5 and it is also secreted by human osteosarcoma cell line (253).

(f) **IGF-binding protein-6 (IGFBP-6)**

IGFBP-6 was originally purified from human cerebrospinal fluid, in which it is the predominant form (254). It is also secreted by an SV-40 transformed fibroblast cell line (255) and human lung fibroblast cell line (256). It is also present in rat and human sera (205,257). IGFBP-6 has ten times higher affinity for IGF-II than IGF-I (254). In adult rat, IGF-II is expressed abundantly in neural tissues but is is barely detected in other tissues (52). The presence of IGFBP-6 in the CSF and its selective affinity for IGF-II could suggest a specific regulatory system involving IGF-II and IGFBP-6 in the brain.

B. **STRUCTURE**

The primary amino acid sequences determined from the cloned cDNAs of IGFBPs reveal structural homology among IGFBPs. These are summarized in Table 6.

TABLE 6

COMPARISON OF THE STRUCTURAL CHARACTERISTICS OF IGFBP -1, -2, -3, -4, -5, and -6 IN HUMANS AND RATS

IGFBP	# a.a. in total protein	# a.a. in mature peptide	predicted mol. wt. of matrix peptide	# cysteines	# Potential ASN linkage	RGD sequence	chromosomal location	size of mRNA (kb.)	References
h IGFBP1	259	234	25,274	18	none	yes	7	1-6	179
r IGFBP1	272	247	29,600	18	none	yes		1-6	180
h IGFBP2	328	289	31,300	20	none	yes	2	1.5-1.6	182
r IGFBP2	304	270	29,564	18	none	yes		1.6-1.7	181
h IGFBP3	291	264	28,500	18	3	no	7	2.4	183
r IGFBP3	292	265	28,900	18	4	no		2.6	184
h IGFBP4	258	237	25,970	20	1	no	17	2.6	185
r IGFBP4	254	233	25,700	20	1	no		2.6	
h IGFBP5	272	252	28,428	18	none	no	5	6.0	186
r IGFBP5	272	252	28,553	18	none	no		6.0	
h IGFBP6	224	201	21,461	16	none	no	12	1.3	187
r IGFBP6	241	216	22,847	14	1	no		1.3	

Structural characteristics of the six IGFBPs denoted in the above table were deduced from cDNA clones. cDNAs for various IGFBPs were cloned from human (h) and rat (r) cDNA libraries.

(a) **Cysteine residues**

The most striking similarity in the structure of the IGFBPs is the conservation in the placement of cysteine residues. Each binding protein has cysteine rich region at the amino and carboxy termini of the protein. In all the BPs there are 18 conserved cysteines that are arranaged in approximately the same spatial order in each BP. hIGFBP-2 (207) and h/r IGFBP-4 (210) have 20 cysteines whereas hIGFBP-6 has 16 and rIGFBP-6 has 14 cysteine residues (212). Conservation of the spatial order of the cysteines indicates that the secondary structure of the IGFBPs is dependent upon disulfide bonding and must be well conserved. Formation of an IGF binding site in the molecule is dependent upon disulfide bonding within the IGF-BP molecule as IGF fails to bind to BPs which are reduced (208).

(b) **Arginine-Glycine-Aspartic acid (RGD) sequence**

Analysis of the amino acid sequences of the IGFBPs reveals the presence of a conserved RGD sequence near the carboxy terminus of IGFBPs -1 and -2. The RGD sequence has been shown to be the minimum sequence in many extracellular matrix proteins required for their binding to membrane receptors of the integrin protein family (259). The hypothesis that IGFBPs may be bound to the cell membrane through their RGD sequences is supported by the observation that a synthetic RGD sequence blocked the binding of hIGFBP-1 to cell membranes (260).

(c) **Differences among IGFBPs**

The most significant difference among the IGFBPs may be in their glycosylation state. IGFBPs -3, -4 and -6 are glycosylated through N-linked carbohydrates. The significance of the glycosylation is unknown, but it may be related to the fact that IGFBP-3 is the major IGFBP in serum and is the only one bound to the acid-labile subunit within the 150 K serum complex. The presence of different glycosylated forms in human and rat serum suggests that there are separate populations of IGFBP -3 and -4 in serum, with possible different functions. IGFBP-3 in the serum of pregnant women is cleaved proteolytically resulting in smaller subunits that appear to have lower binding affinities for IGFs (261).

The homology between human and rat IGFBPs is greater than 70%. When the sequences of different IGFBPs are compared within the same species, the homologies are greater than 50% suggesting that, although they probably share a common genetic origin, they have diverged structurally, perhaps reflecting different functional roles. Furthermore none of the IGFBPs share significant homology with either of the high affinity IGF receptors (202) indicating that they are not derived from a common genetic origin.

C. **BIOSYNTHESIS**

(a) **Genomic organization of IGFBPs**

(i) **IGFBP-1**

IGFBP-1 gene structure has been determined in man (262,263)

and its promoter has also been characterized (264). It is a single copy gene which spans 5.2 kilobases, has a single transcription start site and contains four exons. The first exon and 5' flanking region are highly GC rich and located in CpG island. The CpG island encloses the CAAT box, the TATA box, the transcription start site and a potential Sp1 transcription factor binding site. The CCAAT box region has been demonstrated to be the major cis element involved in basal IGFBP-1 promoter activity in HEP G2 cells (264). The liver factor B1 is the major trans-acting factor stimulating basal IGFBP-1 promoter activity in HEP G2 cells.

(ii) **IGFBP-2**

The rat IGFBP-2 gene has been shown to span 8 kb and consists of four exons, each of which contains protein-coding sequences (265). The amino acid sequences of exons 1, 3 and 4 are 32-50% identical to the corresponding exons of human IGFBP-1 and IGFBP-3 and 87-91% identical to those of human IGFBP-2. Exon 2 of IGFBP-2 shows negligible homology to exon 2 of IGFBP-1 and IGFBP-3.

Unlike the IGF3P-1 (264) and IGFBP-3 (266) genes, the rat IGFBP-2 gene lacks a TATA box near the transcription initiation site. TATA boxes are thought to be important for the accurate initiation of transcription. The TATA box is recognized by the transcription factor TFIID, which forms part of the initial complex with RNA polymerase II (267). Like other TATA-less

genes, including that for the EGF receptor (268) and insulin (269), the IGFBP-2 gene is highly GC rich in its 5' flanking region and in exon 1. This suggests it might be recognized by Sp1 (270) and/or ETF (271). A potential Sp1 site is also present in 5' region of the IGFBP-2 gene.

Like the IGFBP-3 gene (266), but unlike the IGFBP-1 gene (264) the rat IGFBP-2 gene does not have a CCAAT (272) box near start of transcription. But there are potential cis regulatory elements for AP-1, AP-2 and liver factor-B1 present. An AP-1 site can be recognized by jun/jun and jun/fos dimers (273). Most AP-1 proteins are regulated by phorbol esters (273). Transactivating factors that recognize the AP-2 sites are regulated by cAMP and phorbol esters (274). An AP-2 site is also present in the human IGFBP-3 gene (266) but not in the human IGFBP-1 gene (264).

(iii) **IGFBP-3**

The human IGFBP-3 gene has been shown to span 8.9 kilobases. Four exons contain the protein coding regions whereas the fifth exon contains a 3'-untranslated region (266). The size and sequence of exon 2 differ significantly between human and porcine IGFBP-3. These observations suggest that exon 2 encodes for structural and/or functional characteristics conserved among IGFBP-3 proteins from different species but not present in IGFBP-1 (263) or IGFBP-2 (265). Exon 2 encodes that protein region of the IGFBP-3 sequence which becomes glycosylated. It is also possible that exon 2 of IGFBP-3 encodes a sequence

which functions to bind the acid-labile glycoprotein subunit in the 150 K IGFBP-3 serum complex. There is a single copy of the IGFBP-3 gene in the human genome. Its promoter region, cap site, and first exon are all contained within a CpG island. Most CpG islands in the human genome appear to be located at the 5' end of transcription units; the promoter region of these genes is usually included in the CpG island and frequently contains a GC box promoter element. CpG islands appear to bind protein factors which may well influence the ability of gene promoters to regulate transcription rates (275). The region that includes a GC box is likely to bind the transcription factor Sp1 (275). There is also a cis-element present which is known to bind the transcription factor AP-2 (274).

D. **PRODUCTION OF IGFBPs**

(a) **In biological fluids**

The different IGFBPs found in various biological fluids in both human and rat are summarized in Table 7.

(i) **Serum**

The two major IGFBPs in human serum are M_r = 150,000 (large complex) and M_r = 40,000 (small complex). Five molecular forms of IGFBPs of M_r = 41,500, 38,500, 34,000, 30,000 and 24,000 were detected on Western ligand blotting in human serum (231). In normal serum the 41,500 and 38,500 M_r forms were the major binding proteins. They appeared in both complexes, but were

Table 7

IGFBPs in biological fluids

BIOLOGICAL FLUID	BP - 1	BP - 2	BP - 3	BP - 4	BP - 5	BP - 6	REFERENCES
Human serum	+	+	+	+	+	+	229, 210, 211, 212, 257
Rat serum	+	+	+	+	+	+	210, 211, 212, 287, 276, 294
Porcine serum	+	+	+	+	+	+	281, 295
Human lymph	+	+	+	+	+		282
Human CSF	+	+	+		+	+	283
Human amniotic fluid	+		+				286
Rat amniotic fluid		+	-				287
Human follicular fluid	+	+	+				288
Pig ovarian follicular fluid		+	+	+	+	+	212
Seminal plasma	+	+		+			291
Rat milk	+	+	+				293

Various IGFBPs are present in biological fluids. Serum appears to contain all six IGFBPs, whereas other fluids contain only specific IGFBPs.

predominantly in the large complex, where they constitute the elementary binding units. The 34,000, 30,000 and 24,000 M_r forms were visible only in the small complex. Different molecular forms of IGFBPs reveal different affinities for the IGFs. The 41,500 and 24,000 M_r have high affinity for IGF-I whereas the 38,500, 34,000 and 30,000 M_r proteins preferentially bind IGF-II. Recently it has been reported that IGFBP-1 and IGFBP-3 preferentially bind IGF-I whereas IGFBP-2 and IGFBP-4 preferentially bind IGF-II (206). 62 % of immunoreactive IGF-I is bound to the 150 K complex whereas 38 % is bound to the 40 K complex and none is present as free 7.5 K IGF-I. Rat serum contains unoccupied binding sites for IGFs that also are predominantly (77 %) located in the 150 K region and have preferential binding affinity for IGF-II (276).

The existence of a large IGFBP complex and a small IGFBP complex have been demonstrated in adult rat serum (277). The large BPs have been shown to predominate in adult serum, while the small BPs predominate in fetal serum (278).

Fetal and postnatal sera of the pig have been found to contain six molecular forms of IGFBPs, with M_r of 220,000, 43,000, 39,000, 34,000, 29,000 and 24,000 (279,280). The serum concentrations of all six BPs appear to increase with gestational age. In the fetal pig the M_r = 34,000 and M_r = 29,000 forms predominate, while postnatally the major forms are M_r = 43,000 and M_r = 39,000.

Diurnal variation

Serum IGFBP-1 levels have a marked diurnal rhythm and appear to be unaffected by the GH status of the patient or by pulsatile GH secretory activity. Plasma levels of IGFBP-1 can vary 10 to 20-fold over the course of a day, with levels achieving peak values of 50-500 ng/ml at 8.00 A.M. This diurnal variation was inversely related to plasma insulin levels (281).

(ii) **Lymph**

IGF concentrations in lymph are 10-30% of the corresponding serum levels and the ratio of IGF-I to IGF-II is similar to that in serum. Five molecular forms of IGFBPs have been identified but in significantly smaller concentrations (282). Less than 10% of the binding activity of lymph was found complexed to the large M_r = 150,00 IGFBP and 90% of the IGF binding activity was the smaller IGFBP which had preferential affinity for IGF-II. M_r = 150,000 IGFBP is not capable of crossing the capillary barrier and might act to retain IGF peptides within the vascular compartment. On the other hand, the M_r = 34,000, 30,000 and 24,000 molecular forms appear capable of crossing the capillary barrier and might be involved in transporting IGFs to their target cells.

(iii) **Cerebrospinal fluid (CSF)**

Hossenlopp et al (283) demonstrated five molecular forms of IGFBPs in human CSF. These BPs ranged from M_r = 24,000 to

41,000 with the major BP at M_r = 34,000. This BP resembled IGFBP-2 (284). and had higher afinity for IGF-II than IGF-I. IGFBP-5 (251) and IGFBP-6 (254) have also been identified in CSF and they also have higher affinity for IGF-II than IGF-I.

(iv) **Amniotic fluid**

Amniotic fluid has been shown to be a rich source of IGFBP-1 termed "amniotic fluid BP" (285). Baxter et al (286) further demonstrated the presence both BP-28 (IGFBP-1) and BP-53 (IGFBP-3) in amniotic fluid and showed that levels of both BPs decline with increasing fetal maturity. The rat equivalent of IGFBP-1 has not yet been identified in rat amniotic fluid. The major amniotic fluid IGFBP in rat is IGFBP-2 (287).

(v) **Follicular fluid**

The existence of PP12 (IGFBP-1) has been demonstrated in human follicular fluid (288). Porcine follicular fluid has been shown to contain IGFBP-1, -2, -3, -4, -5 and -6 (289,212). The significance of IGFBPs in follicular fluids is not clear.

(vi) **Seminal plasma**

Seppala et al (290) found IGFBP-1 like immunoreactivity in human seminal plasma. The levels were similar to those found in normal male serum. IGFBP-2 and IGFBP-4 were also detected in human seminal plasma (291). The source of seminal plasma IGFBPs remains to be elucidated.

(vii) **Milk**

The presence of growth factors in breast milk could be important for the proliferation and maturation of the intestinal epithelium. They are also absorbed intact from the gut and transferred to the infant.

The presence of IGFBP-1 in human milk was demonstrated by Suikkari (292). A positive correlation was identified between the milk level of IGFBP-1 and IGF-I. IGFBP-1, -2 and -3 have been identified in rat milk on days 5-17 of lactation (293).

(b) **Fibroblasts**

Rat embryo fibroblasts produce IGFBP-2 in their culture medium (296). Neonatal human skin fibroblasts were found to produce two IGFBPs which resemble plasma IGFBP-3 (297). Conover et al (247) using Western ligand blotting, showed that the conditioned medium of human fibroblasts contained five molecular forms of IGFBPs of M_r = 41,500, 37,000, 32,000, 28,000 and 23,000, resembling the BPs found in human serum (229).

(c) **Ovary**

IGFBP-1 is produced by human granulosa cells (298) and is also detected in human follicular fluid (288,299). Purified IGFBP-3 and IGFBP-2 from pig ovarian follicular fluid inhibited FSH and cAMP stimulated estradiol and progesterone production in cultured rat granulosa cells (300). This inhibition by IGFBPs could be the result of the combination of two effects:

(a) sequestration of endogenously produced IGF-I and (b) direct interaction with the cell to elicit the inhibition.

Both rat and porcine granulosa cells produce IGF-I and IGF-II and the ovary is a target for insulin and IGF action (301). IGF receptors have also been detected in human ovarian follicles (302). All the components for autocrine/paracrine action (i.e. IGF-I, IGF-II, IGF-receptor and IGF-binding proteins) are present in human granulosa cells. It is likely that BPs participate in the regulation of the cellular growth response to IGF-I.

(d) **In the Central Nervous System (CNS)**

Rat astroglial cells produce IGFBP-2 (303) and the rat neuroblastoma cell line, B 104, secretes two IGFBPs of M_r = 36,500 and M_r = 39,000 (304). Conditioned medium from primary cultures of rat anterior pituitary cells contained IGFBPs with apparent M_rs of 35,000, 27,000 and 24,000 (305). M_r = 35,000 IGFBP is glycosylated and resembles IGFBP-3. M_r = 27,000 is nonglycosylated and resembles IGFBP-2. Rat astrocytes, fetal rat neuronal cells and neurointermediate pituitary produce IGFBP-2 (284). Rat astrocyte cultures also produce IGFBP-3. B 104 neuroblastoma cells produce two smaller BPs with M_r = 26,000 (minor band) and M_r = 23,000 (major band). The presence of IGFBP-2 in the rat central nervous system is further documented by Northern blots which show that IGFBP-2 mRNA is present in cultured fetal neurons, cultured newborn astroglial

cells, adult rat brain, pituitary and hypothalamus and fetal rat brain (284).

The role of IGFBPs in the physiology of the pituitary and central nervous system remains uncertain. It is clear that BPs can modulate the access of IGFs to the pituitary and the hypothalamus, thereby potentially influencing feedback control of GH production and secretion (306). They may also act as carriers for IGFs from the vascular compartment to IGF receptors (307).

E. REGULATION OF IGFBPs PRODUCTION AND GENE EXPRESSION

(a) IN VIVO

(i) Tissue specific

IGFBP mRNA levels

IGFBPs are ubiquitous proteins and their mRNAs are expressed in various adult and fetal rat tissues. Various tissues expressing IGFBP mRNAs are listed in Table 8. IGFBP-1 mRNA levels are not detected in human fetal tissues other than liver (204). Fetal rat tissues express high levels of IGFBP-2 mRNA levels whereas IGFBP-3 mRNA levels appear postnatally.

IGFBP-1 and IGFBP-2 mRNA abundance in fetal rat tissues are shown in Table 9 (310). Although the relative abundance of the two mRNAs were similar in most fetal rat tissues examined, IGFBP-1 mRNA was 8 fold less abundant than IGFBP-2 mRNA in kidney and more than 25 fold less abundant in brain. This may indicate specific roles of IGFBP-2 in these tissues (310). Although IGFBP-2 mRNA expression is decreased in most

Table 8

IGFBPs mRNAs are expressed in various adult and fetal rat tissues. High abundance of IGFBP-2 mRNA is present in fetal tissues than in adult tissues. IGFBP-4, -5, -6 are mainly expressed in adult tissues and are barely detected in fetal tissues.

Table 8

Tissue distribution of IGFBP mRNAs in adult and fetal rat tissues

TISSUES	BP-1	BP-2	BP-3	BP-4	BP-5	BP-6	REFERENCES
ADULT RAT							
Adrenal				+	+	+	
Testis		+		+	+	+	
Spleen				+	+	+	
Heart				+	+	+	
Lung				+	+	+	
Kidney	+	+		+	+	+	
Liver	+		+	+	+		
Stomach			+	+	+	+	
Hypothalamus				+			
Brain	+	+		+	+	+	
Intestine					+	+	
Uterus	+		+				
Placenta	+		+				
Ovary	+	+	+	+	+		287, 288
FETAL RAT							
Liver	+	+					
Kidney		+					
Intestine		+					
Lung		+					
REFERENCES	180	181	184	185	186	187	

Table 9

Tissue specific regulation of IGFBP-1 and IGFBP-2 mRNA levels in fetal rat

TISSUE	IGFBP-1 mRNA LEVELS	IGFBP-2 mRNA LEVELS
Liver	++++++++	++++++++
Stomach	++	++++
Kidney	+	++++
Lung	++	++
Intestine	-	-
Muscle	-	-
Heart	++	+
Skin	+	+
Brain stem	-	+++++ +
Cerebral cortex	-	++++++
Hypothalamus	-	+++++

IGFBP-1 and IGFBP-2 mRNAs are expressed in various fetal tissues. The abundance of BP mRNAs is indicated by the number of pluses (+). Fetal liver was found to contain more IGFBP-1 mRNA than other fetal tissues. No IGFBP-1 mRNA was detected in fetal brain. Fetal liver and brain contain higher levels of IGFBP-2 mRNA than other tissues. Taken from reference 310.

nonneuronal tissues of the adult rat. IGFBP-2 mRNA is expressed in choroid plexus of adult rat brain and IGFBP-2 is the principal IGFBP in cerebrospinal fluid.

(ii) **Developmental**

In the rat serum

The two BPs of M_r = 150,000 and M_r = 40,000 are present in rat serum (311). The large BP predominates in adult serum whereas the small BP is IGFBP-2 (312). Seven molecular forms of BP were identified by Western ligand blotting, with apparent M_rs of 42,000, 41,000, 40,000, 38,000, 28,000, 26,000 and 22,000. Following deglycosylation with endo F, the four largest bands were reduced in size to two bands with apparent M_rs = 35,000 and 32,000; the M_r = 28,000, 26,000 and 22,000 were found in neonatal rat serum with the M_r = 28,000 BP decreased in amount. The four M_r = 38,000-42,000 BPs appeared at approximately 19 days of age. M_r = 28,000 BP was found to be IGFBP-2. IGFBP-3 appeared during the third week of life and gradually increased to adult level, concomitant with an increase in serum IGF-I and a decrease in serum IGF-II concentrations. The developmental factor(s) that induces the synthesis of the adult binding complex, while suppressing expression of the neonatal BP, is unknown. One theory is that the shift may be caused by the increasing responsiveness of the newborn rat to the action of GH during the second and the third postnatal week (313) (GH → IGF-I → IGFBP-3). IGFBP-1 and IGFBP-2 mRNAs are developmentaly regulated, being expressed at

a higher levels in fetal and neonatal rat liver than in adult liver (Table 10) (310). The developmental pattern observed with IGFBP-1 and IGFBP-2 mRNAs parallels the pattern observed with the serum levels of IGFBP-1 and IGFBP-2.

In human serum

Unsaturated IGFBP activity of M_r = 40,000-50,000 was elevated in newborns. The IGFBP-1 level was elevated in fetal blood, fell significantly at birth and declined further throughout childhood until adult life (285). It is not clear at this time whether the major serum BP in the fetus/newborn is IGFBP-2, IGFBP-1 or both. The serum level of IGFBP-3 rises progressively during childhood thus paralleling the increase in serum IGF-I levels. The values of IGFBP-1 showed a gradual decline with increasing age. Fetal serum was found to contain 36.7 ug eq/ml compared to adult serum which contained 0.6 ug eq/ml. These changes are reflected at the mRNA level, as IGFBP-1 mRNA levels were higher in liver from 14 to 16 weeks old human fetuses than in adult liver (204).

(iii) **In fasting and GH deficiency**

Serum levels of IGF-I, IGF-II and IGFBP-3 are reduced by poor nutrition, whereas the levels of IGFBP-1 and IGFBP-2 are increased (314). In human serum, IGFBP-1 is increased 4 to 12 fold during fasting (315) and 2 fold in GH deficiency (315). Neonatal rats on restricted caloric intake have elevated levels

Table 10

Developmental regulation of IGFBP-1 and IGFBP-2 mRNA levels in fetal and adult rat liver

PRENATAL OR POSTNATAL AGE OF THE RAT	IGFBP-1 mRNA LEVEL	IGFBP-2 mRNA LEVEL
21 day gestation	++++++++	++++++++
1 day postnatal	++++++++	+++++++++
21 day postnatal	++	+
65 day-old-adult	+	-

Rat liver, at 21 days gestation and 1 day postnatal contains a high level of IGFBP-1 and IGFBP-2 mRNAs. BP-1 and BP-2 mRNA levels decline with increasing age. The abundance of BP-1 and BP-2 mRNAs is expressed by the number of pluses (+). Taken from reference 310.

of IGFBP-2 (314). There is a delay in the developmental switch from IGFBP-2 to IGFBP-3 which normally occurs between day 15 and 19 postnatally. Refeeding normalizes IGFBP-2 levels. Since IGFBPs are inhibitory for IGF action (316), the reduction in serum IGF peptide coupled with increased serum IGFBP may work together to limit growth so that nutrients are used for metabolism. Changes in the circulating IGFBP patterns during malnutrition are in part at RNA level. In one study, no change in hepatic IGFBP-1 mRNA was observed in fasted or hypophysectomized rats whereas IGFBP-2 mRNA was increased 10 to 12 fold in fasting and hypophysectomy and was accompanied by an increase in circulating IGFBP-2 (310). GH treatment failed to normalize IGFBP-2 mRNA in hypophysectomized rats. GH plus cortisone acetate plus T_4 plus testosterone decreased hepatic IGFBP-2 mRNA by 57% relative to that in hypophysectomized rats (310). A similar study carried out by others, reported an increased level of hepatic IGFBP-1 mRNA in fasted rats followed by an increase of IGFBP-1 in serum (317). Hypophysectomy also resulted in an increased level of hepatic IGFBP-1 mRNA and circulating levels of IGFBP-1 in serum (318). Elevated hepatic mRNA and serum levels of IGFBP-1 are normalized by refeeding (317) and by GH treatment respectively (318).

Hepatic IGFBP-3 gene expression is partly GH dependent with IGFBP-3 mRNA levels falling after hypophysectomy and rising slightly after GH treatment (319). In contrast, renal IGFBP-3 mRNA increased after hypophysectomy but did not decrease with GH treatment. These data suggest that IGFBP-3 mRNA abundance is

regulated differently in different tissues (319). The exact mechanism(s) mediating the differential regulation of IGF peptide and IGFBPs remains to be defined. The fasting induced decrements in IGF levels, as well as the elevation of IGF-associated binding proteins may serve a protective role to depress growth at times of caloric restriction.

(iv) **In Diabetes**

Serum insulin concentration plays a role in the regulation of serum IGFBP-1 levels (320). IGFBP-1 levels are elevated in type 1 and type 2 diabetic patients and decrease in patients with insulinoma. IGFBP-1 is decreased by acute hyperinsulinemia (320).

IGFBP-3 is lacking in diabetic rats. This subunit is induced by insulin treatment and appears in the 150-200 kDa complex. Infusion of recombinant human IGF-I (rhIGF-I) induces the IGFBP-3 subunit and the formation of the 150-200 kDa complex in diabetic rats. but rhIGF-I stimulates IGFBP-3 in hypophysectomized rats but does not promote formation of the form 150-200 kDa complex (321). Significant accumulation of this 150-200 kDa complex occurs only in the presence of GH (321). It is likely that GH but not IGF-I induces a component, which itself does not bind IGF but associates with the glycosylated IGF binding subunit. The glycosylated subunit protects an individual against IGF-induced hypoglycemia and it may be involved in tissue specific targeting of IGFs. Baxter

(322) showed that the synthesis of this acid-labile component is critically dependent on GH, but is not induced by IGF-I.

IGFBP-1 levels were elevated in the sera of diabetic rats and reached control levels with insulin treatment. Levels were again elevated after insulin was withdrawn (323). Similar changes of hepatic IGFBP-1 mRNA levels were also observed in diabetic rats (310).

IGFBP-1 mRNA is increased 100 fold in livers of streptozotocin-induced diabetic rats (315) and a similar increase of IGFBP-1 was observed in diabetic rat serum. IGFBP-2 mRNA was slightly increased in severely diabetic rats. Insulin treatment did not normalize IGFBP-2 mRNA (289). Boni-Schnetzler et al (324) reported that although the abundance of hepatic mRNA for IGFBP-2 may rise rapidly during insulin deficiency, normalization may require extended insulin therapy, perhaps due to the prolonged survival of these mRNAs.

Insulin stimulated production of IGF-I and IGFBP-3 could stabilize serum levels of IGF-I and protect an individual from the hypoglycemic effect of IGFs. Regulation of low mol wt IGFBPs by insulin and perhaps other factors, may play an important role in the modulation of tissue growth factor bioactivity in metabolic disease states.

(v) **Dexamethasone**

Dexamethasone increases hepatic IGFBP-1 mRNA levels 10 fold and also stimulates serum IGFBP-1 levels in rats (325). If IGFBP-1 functions to decrease the bioavailability of IGF-I in

vivo (326), the enhanced expression of IGFBP-1 may be an additional mechanism whereby glucocorticoid excess results in growth retardation.

(vi) **Cancer**

Increasing serum levels of IGFBP-1 has been reported in patients with trophoblastic disease and ovarian cancer (327). The role of IGFBP-1 in proliferation or progression of cancer is not known.

(b) **IN VITRO**

(i) **In various cell types in culture**

The observations made by several investigators on the regulation of IGFBP mRNA expression and peptide production in various types of cultured cells have been summarized in Table 11. IGFBP-I is mostly expressed in liver cells whereas IGFBP-3 is produced by fibroblasts and epithelial cells. Epithelial cells also produce IGFBP-2 and osteroblasts and neuroblastoma cells produce IGFBP-4.

As indicated in Table 11, various hormones and agents that stimulate IGFBP-1 production appear to inhibit IGFBP-3 production. Insulin, IGF-I and PMA inhibit IGFBP-1 and IGFBP-2 mRNA levels and their respective peptide production in hepatocytes whereas they stimulate IGFBP-3 mRNA levels and IGFBP-3 peptide production in fibroblasts. Dexamethasone and cAMP stimulate IGFBP-1 mRNA levels in hepatocytes and in H4IIE

Table 11

Regulation of IGFBPs production and expression of their mRNAs in cells in culture

Cells	Effector	IGFBP-1 mRNA	IGFBP-1 peptide	IGFBP-2 mRNA	IGFBP-2 peptide	IGFBP-3 mRNA	IGFBP-3 peptide	IGFBP-4 mRNA	IGFBP-4 peptide	References
Rat hepatocytes	insulin	↓	↓							331, 231
	insulin			↓	↓					332
	dexamethasone	↑								- 331
	glucagon	↑								
	bGH	↓								
	T_3	↓								
	cAMP	↑								
	PMA	↓								
	IGF-I			↓						332
H411E rat hepatoma cells	dexamethasone	↑	↑							333
	progesterone	→	→							333, 230
	testosterone	→	→							333, 230
	IGF-I	→								333
	rGH	→	→							333, 230
	insulin	↓	↓							333, 334
	cAMP	↑	↑							230
	estradiol	→	→							230
Fetal rat liver explant	cycloheximide		↓							335
	theophyllin		↑							
	glucagon		↑							
	forskolin		↑							
	glucose		↓							
	dexamethasone		↓							
	insulin		↓							

TABLE 11 (CONT'D)

Cells	Effector	IGFBP-1 mRNA	IGFBP-1 peptide	IGFBP-2 mRNA	IGFBP-2 peptide	IGFBP-3 mRNA	IGFBP-3 peptide	IGFBP-4 mRNA	IGFBP-4 peptide	References
Bovine fibroblasts	IGF-I insulin					↑ ↑	↑ ↑			310
Human skin fibroblasts	TGF-∝ FCS						↑ ↑			311
Adult human fibroblasts N3652	IGF-I IGF-II insulin						↓ ↓ ↑			312
Swiss 3T3 cells	untreated bombesin vasopressin PDGF EGF PMA IGF-I cAMP dexamethasone insulin					↑ →	↑ ↑ ↑ ↑ ↑ ↑ ↑ ↓ ↓ →			313
Bovine epithelial cells	untreated forskolin			↑ ↓	↑ ↓	↑	↑			314
Bovine mammary epithelial cells	untreated IGF-I				↑ ↑		↑ ↑			315

Table 11 (cont'd)

Cells	Effector	IGFBP-1 mRNA	IGFBP-1 peptide	IGFBP-2 mRNA	IGFBP-2 peptide	IGFBP-3 mRNA	IGFBP-3 peptide	IGFBP-4 mRNA	IGFBP-4 peptide	References
Rat osteoblast like cells (ROB)	untreated			↑	↑				↑	341
	estradiol (low dose)				↓					
	estradiol (high dose)				↑					
	hGH				↑				↑	
Mouse osteoblast	1, 25, $(OH)_2$ Vit D_3							↑	↑	342
Human osteosarcoma cells MG 63	1, 25, $(OH)_2$ Vit D_3						↑			343
Sertoli cells	FSH						↓			344
	isopreterenol						↓			
	cAMP						↓			
	choleratoxin						↓			
	IGF-I						↑			
Sheep thyroid cells	untreated			↑	↑					345
	EGF					↑	↑			
	phorbol esters					↑	↑			

Table 11 (cont'd)

Cells	Effector	IGFBP-1 mRNA	IGFBP-1 peptide	IGFBP-2 mRNA	IGFBP-2 peptide	IGFBP-3 mRNA	IGFBP-3 peptide	IGFBP-4 mRNA	IGFBP-4 peptide	References
Rat neuroblastoma cell line B 104	IGF-I IGF-II						↑ ↑		↑ ↓	346
Human epidermal squamous cell carcinoma line SCL-1	IGF-I IGF-II insulin						↑ ↑ ↑		↑ ↑ ↑	337
Human decidual cells	untreated cAMP hydrocortisone PMA		↑ ↑ → →		↑ → → →				↑ ↓ →	347

Various cell types secrete IGFBPs -1, -2, -3 and -4 and express their mRNAs. Several hormones, growth factors or agents, stimulate (↑), inhibit (↓) or have no effect (→) on IGFBPs secretion or their mRNAs expression in these cells.

hepatoma cells, and their peptide production in H4IIE cells. They inhibit IGFBP-3 mRNA levels in Swiss 3T3 cells.

Human breast cancer cell lines (HBCC) secrete at least four different forms of IGFBPs (328). The human breast cancer cell line Hs578T has been shown to produce IGFBP-1, BP-3 and BP-4 and express mRNAs for BP-1 and BP-3. MCF-7 cell line secretes IGFBP-2 in its culture medium and expresses mRNA for BP-2. MDA-231 cells secrete IGFBP-3 (248). No IGF-I or IGF-II have been detected in these cells (328). HBCC do not secrete IGF-I or contain mRNA for this peptide (329). HBCC respond to nanomolar concentrations of IGF-I and IGF-II and secrete considerable amount of IGFBPs. IGF peptides may interact in a paracrine fashion to promote the growth of human breast carcinomas (329). Since the IGF-I/BP complex can be a better mitogen than free IGF-I (330) and IGFBPs can modulate IGF actions, secretion of IGFBPs by HBCC may regulate cellular responses to IGFs.

(ii) **In cultured rat hepatocytes**

Primary cultured rat hepatocytes express IGFBP-1 and IGFBP-2 mRNAs and secrete IGFBP-1 and IGFBP-2 peptides into their culture medium. Insulin has been shown to inhibit IGFBP-1 and -2 mRNA and peptide production in hepatocytes. IGF-I, at a pharmacological concentration, also inhibits IGFBP-2 mRNA levels.

Our studies showed that insulin, bGH and T_3 each inhibited IGFBP-1 mRNA levels. Insulin was found to be the most powerful

inhibitor and inhibited IGFBP-1 mRNA levels 70% of control. bGH was slightly less effective and inhibited IGFBP-1 mRNA levels 50 % of control. Dexamethasone and glucagon each stimulated IGFBP-1 mRNA levels 4 to 6 fold and had an additive effect on IGFBP-1 mRNA stimulation suggesting that they act via different mechanisms. Insulin inhibited IGFBP-1 mRNA levels in the presence of dexamethasone and glucagon.

The mechanisms of glucagon and bGH effect on IGFBP-1 mRNA levels were further investigated as these hormones have not been reported to have any effect on IGFBP-1 mRNA levels in hepatocytes. Like glucagon, IBMX and $(Bu)_2$cAMP stimulated IGFBP-1 mRNA levels. An inhibitory effect of bGH on IGFBP-1 mRNA level was prevented in protein kinase C (PKC) depleted cells and also in the presence of inhibitors of protein kinases. bGH was found to superinduce IGFBP-1 mRNA in the presence of cycloheximide suggesting the presence of a repressor protein that inhibited IGFBP-1 mRNA levels. bGH appears to stimulate the synthesis of this protein.

The effect of bGH appeared to be exclusively at the level of transcription to inhibit IGFBP-1 mRNA levels, whereas that of glucagon and $(Bu)_2$cAMP were in part at a postranscriptional level as well to stabilize IGFBP-1 mRNA. Thus hepatic IGFBP-1 mRNA is multihormonally regulated and its expression in vivo may depend on the physiological status of the animal.

F. **BIOLOGICAL ACTIONS**

The ability of these proteins to bind specifically to IGF peptides with high affinity leads to the hypothesis that the BPs act to modulate the actions of the IGFs by regulating their access to target cell membrane receptors. The BPs may also have some kind of regulatory or effector actions, independent of IGFs. The presence of multiple IGF-BPs expressed differentially in various tissues suggests that each BP species could have a specific regulatory role(s). The experimental evidence to date supports at least three different roles for IGFBPs.

(a) **IGFBP-1**

IGFBP-1 has been shown to both inhibit and enhance the mitogenic actions of IGFs in different cell types. Purified preparations of IGFBP-1 appear to compete with membrane receptors in endometrial tissue and placenta for the binding of IGF-I (348,349). Purified human IGFBP-1 was also shown to block the binding of IGF-I to its membrane receptors on the choriocarcinoma cell line, JEG-3, and inhibit IGF-I stimulated uptake of amino acids by these cells (350). These studies have suggested that one action of IGFBP-1 might be to inhibit IGF action by directly competing with membrane receptors.

An enhancing effect of IGFBP-1 has been reported in the stimulation of DNA synthesis in porcine aortic smooth muscle (351), fibroblast (352), and FRTL 5 thyroid cells (353). Stimulation of these cells by IGF-I in the presence of human IGFBP-1 resulted in a 2 to 4 fold greater synthesis of DNA than

that observed with IGF-I alone.

(b) **IGFBP-3**

IGFBP-3 appears to be a carrier for IGF peptides in serum and possibly, in other biological fluids. IGFBP-3 is involved in a M_r = 150,000 complex. The binding of IGF peptide to this complex significantly increases the half life of the peptide (354). It has been suggested that IGFBP-3 protects circulating IGF peptide from degradation and thereby increases the amount of IGF potentially available to target tissues. It remains unclear, however, as to whether IGF peptides bound to IGFBP-3 are capable of being delivered to target tissues or whether they remain unavailable complexed within the serum. It has been reported that the mitogenic activity of IGF-I in baby hamster and human fibroblasts was enhanced when IGF-I was first complexed to purified human IGFBP-3 (330).

IGFBP-3 in the microenvironment has the potential to alter directly cellular responses to IGFs. Both inhibiting and enhancing effects of IGFBP-3 on IGF-I action have been reported (355). Inhibition appears to result from soluble IGFBP-3 sequestering IGF-I and preventing receptor interaction (356). Cultured human fibroblasts preexposed to purified IGFBP-3 showed increased responsiveness to IGF-I stimulation (245). This potentiation of IGF-I action was related to IGFBP-3 cell association (245).

G. **STRUCTURAL CHARACTERISTICS OF IGFBP-1**

(a) **Phosphorylated form**

Human amniotic fluid and human fetal serum contain a large proportion of non-phosphorylated IGFBP-1, as well as the phosphorylated form (357). In contrast, HEP G2 cells and human decidual cells secrete predominantly the phosphorylated isoforms. These obeservations suggested that IGFBP-1 is secreted as a phosphoprotein and is subsequently dephosphorylated in vivo (357). Phosphorylated IGFBP-1 secreted by Hep G2 cells has 6 fold higher affinity for IGF-I than it does after dephosphorylation. Phosphorylation of IGFBP-1 may be a physiologically important posttranslational modification.

(b) **Importance of Cys-226**

The deletion of the C-terminal 20 amino acids in IGFBP-1 results in loss of IGF binding and may stimulate formation of dimers of IGFBP-1 (358). These dimers are probably formed when cysteine-226 (Cys-226) is missing, and its putative partner is able to form intermolecular disulfide bonds. Site directed mutagenesis demonstrated that most of the introduced point mutations in the C-terminal region did not affect IGF binding. Only mutation of Cys-226 to tyrosine completely abolished IGF binding as did the introduction of negatively charged amino acids in the vicinity of this residue. Again dimers were observed, supporting the view that Cys-226 is essential for the conformation of IGFBP-1. In addition these data suggest that an IGF binding domain may be located in the vicinity of

the intramolecular disulfide bond formed by Cys-226 and its putative partner (358).

III. CIRCULATING FORMS OF IGFS

A. IN PLASMA/SERUM

The principle somatomedins characterized in human serum are IGF-I, a 70 amino acid basic peptide and IGF II, a 67 amino acid acidic peptide (10,11). Various variant forms of IGFs have been reported. Rinderknecht and Humbel (12) found that 25% of the IGF-II derived from human plasma lacked the N-terminal alanine. Jansen et al (46) predicted the existence of an IGF-II peptide in which ser 29 in "Classical" IGF-II was replaced by the amino acids Arg-Leu-Pro-Gly. This has been identified in human plasma (359). Six somatomedin peptides were isolated from Cohn fraction IV (360). Peptides with isoelectric points (PIs) of 9.2, 8.7 and 6.7 were immunoreactive with antisera against IGF-I and those with PIs of 6.3 and 6.15 were reactive with antisera against IGF-II. These variant forms were present in nearly all sera with only minor changes in distribution occuring during childhood and adult life (361).

A 10 kilodalton (KDa) form of IGF-II has been isolated from human plasma known as "big" IGF-II. The major difference observed was the presence of a 21-residue extension on the carboxy end of IGF-II molecule. The amino acid sequence of this extension corresponded to the proximal portion of the E domain of proIGF-II derived from the mRNA of IGF-II (46).

Another larger form of IGF-II was isolated from Cohn fraction II (362). This big IGF-II had an estimated M_r of 15,000. The first 28 amino-terminal residues were identical to IGF-II, except for position 23 and there was evidence of a carboxy-terminal extension. This 15 KDa variant of IGF-II had equal affinity for plasma IGF binding proteins and for the IGF-II receptor of rat plancenta. It also appeared to have even greater mitogenic activity on human fibroblasts than did IGF-I or IGF-II. Normally, these large forms of IGF-II contribute less than 10% to the total IGF-II in human plasma but patients with certain large tumors associated with hypoglycemia may have upto 75% of their serum IGF-II as "big" IGF-II (363).

The possible presence of large forms of IGF-I in human serum has been investigated (364). Sera from normal children and adults were tested by RIA using antibody raised to amino acids 21 to 33 of the E domain of ProIGF 1A (364). RIA of sera from normal children and adults gave negative results, but both children and adults with chronic renal failure had sera which reacted with the antibody. The immunoreactive peptide had an apparent M_r of 19,000. These results indicate that negligible concentrations of ProIGF-I A are present in normal human serum, but proIGF-1A accumulates in the serum in uremia. It is not known whether ProIGF-1B accumulates in a similar manner.

B. **IN BODY FLUIDS**

(a) **Human Urine**

The presence of IGF-I in human urine was reported by Hizuka

et al (365). Urinary IGF-I is found to be GH dependent . Unlike serum IGF-I, which is virtually 100% bound to carrier proteins, 30% of urinary IGF-I is in the free form and 70% is protein bound. Preterm infants excreted more IGF-I than did full term infants and considerably more IGF-I than did older children. Urinary IGF-I levels of infants were correlated with the known concentrations of IGF-I in plasma, which is lowest in preterm infants and rises progressively with age. Albini et al (366) reported that urinary IGF-I of pregnant women rose 4-fold during the first trimester and 10-fold in the third trimester as compared to nonpregnant women. These results contrast with the much more modest rise of serum IGF-I during pregnancy and raises a question about the source of urinary IGF-I. IGF-I has been localized to renal collecting ducts by immunohistochemical staining (367).

(b) **Lymph**

Cohen and Nissley (368) assayed somatomedin bioactivity in rat lymph using chick embryo fibroblasts. They also found that lymph contains about half the activity of rat serum and associated with binding proteins.

(c) **Amniotic Fluid**

The concentration of IGF-I in amniotic fluid was 20 ug/L and that of IGF-II was about 100 ug/L. A decrease in IGF-II concentration occured late in gestation, but IGF-I

concentration remained unchanged (369). Amniotic fluid IGFs could have a role in the growth and maturation of fetal lung and gastrointestinal tract with which they come into contact.

(d) **Cerebrospinal fluid (CSF)**

Human spinal fluid contains 50 ug/L IGF-II and only 2 ug/L IGF-I. About 45% of the immunoreactive IGF-II is present as "Big" IGF-II with an apparent mol wt of about 9,000 (370). CSF IGF concentrations are independent of the GH status (371) of the subject. It is likely that CSF IGF-II arises primarily from the choroid plexus and leptomeninges (372).

(e) **Ovarian Follicular Fluid**

Concentrations of ovarian follicular fluid IGF-I is between 30 and 81% of that of human serum and IGF-II is present in significantly higher concentrations, about 2000 ug/L (373,374).

(f) **Seminal Fluid**

Seminal fluid contains about 20 ug/L IGF-I (375) and 2000 ug/L IGF-II (374). Testis is the predominant source of seminal fluid IGFs. Sertoli cells may be a major source of seminal fluid IGF-I because porcine Sertoli cells secrete IGF-I into the medium (376).

(g) **Human Saliva**

A low concentration of IGF-I (0.2 ug/L) was found in human saliva (377) where it was not bound to IGF-binding proteins.

salivary IGF-I appears to be GH regulated as increased concentrations were found in saliva from patients with acromegaly.

(h) **Human Milk**

IGF-I is present in human milk, in concentration of about 20 ug/L early in lactation and 6-8 ug/L later in lactation (378). 20% of milk IGF is in the free form.

IV. PHYSIOLIGICAL EFFECTS OF IGFS

A. Target Tissues and cells

The target tissues and cells for IGFs are 1. adipocytes 2. heart and skeletal muscles 3. fibroblasts 4. chondrocytes 5. osteoblasts.

B. Receptors

The biological activity of any hormone depends on the ability of the target cell to respond to the signal in extracellular environment and this function is performed by cell surface receptors.

(a) **Characteristics and subtypes**

Affinity-crosslinking studies have revealed the existence of two distinct insulin-like growth factor (IGF) receptors (379,380).

1. Type I IGF receptor

2. Type II IGF receptor

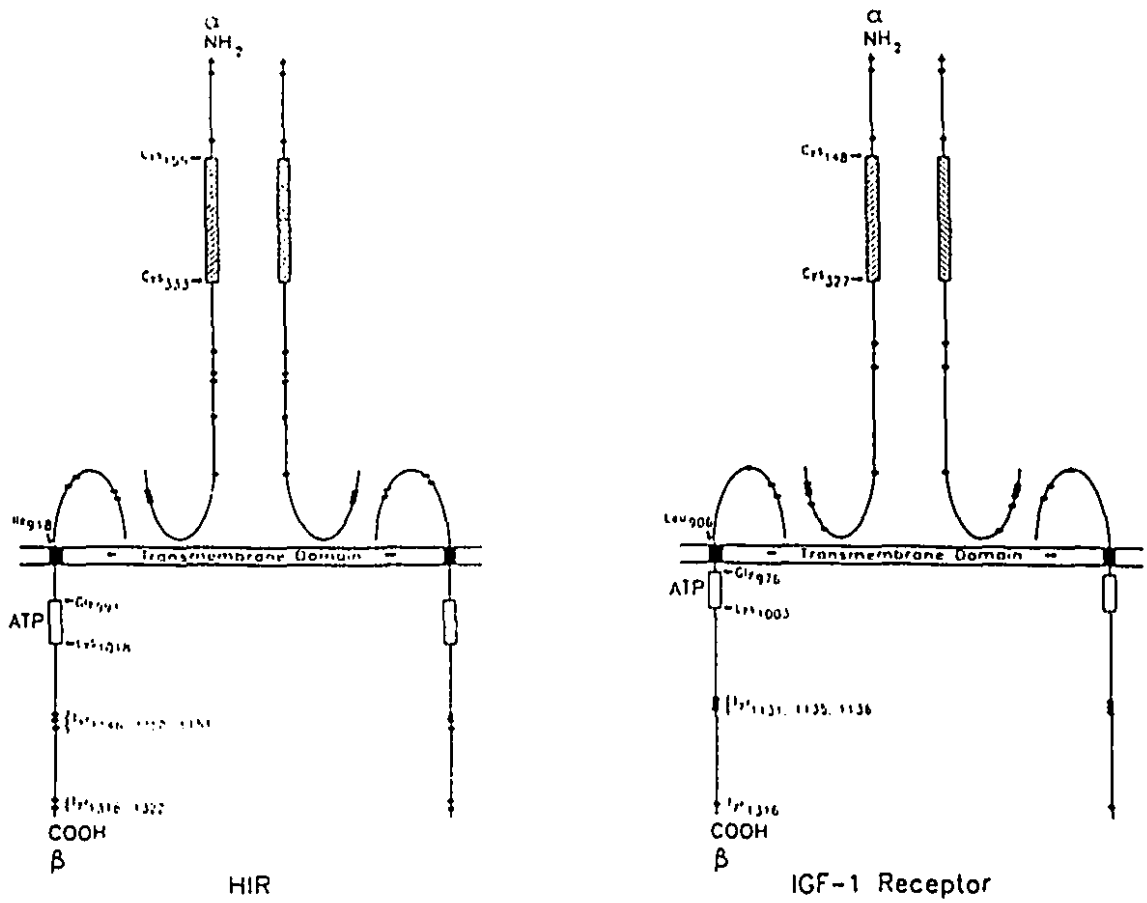
(i) **Type I IGF Receptor and Insulin Receptor**

The type I IGF receptor has structural and functional similarities to the insulin receptor. This receptor generally binds IGF-I with high affinity than IGF-II and also interacts weakly with insulin. Type I and insulin receptors are glycoproteins with molecular weights of between 300 K and 350 K. They consist of two extracellular α-subunits (130K) and two transmembrane ß-subunits (95K) disulfide linked into an heterotetramer ($\alpha_2\beta_2$) (380,381). A schematic model depicting the major structural features of the insulin and Type I receptors is presented in Figure 5 (381a). Small differences in the M_r between the Type I and insulin receptor α and ß subunits in various tissues reflect alterations in posttranslation modification since both receptor species are synthesized from essentially identical α-ß protein precursors of M_r = 151,869 and M_r = 152,784 for Type I and insulin receptors respectively (382,383). The primary structures of the human insulin and Type I receptors have been identified from their respective cDNAs (382,384). Their receptor precursor proteins are glycosylated, dimerized and proteolytically processed to yield α_2-β_2 heterotetrameric receptor complexes (385,386). The human Type I receptor gene has been mapped to chromosome 15.

There is a 50% overall amino acid sequence similarity between insulin and Type I receptors with the highest degree of sequence identity (84%) occuring in the kinase domain

Figure 5

Human insulin (HIR) and IGF-I receptor



Schematic representation of the major structural domains of the human insulin and IGF-1 receptors. The hatched region in the α subunits signifies a cysteine rich domain; individual cysteine residues are denoted by a dark circle. The looped region between the α and β subunits represents a proposed protected hydrophobic pocket in which the class II disulfides are thought to reside. The transmembrane domain is represented by the dark rectangle and the intracellular ATP binding pocket is denoted by the open box. The intracellular tyrosine-specific autophosphorylation acceptor sites are indicated by sequence position and with an open circle. Taken from reference 380a.

(382,384). The largest degree of sequence divergence between these receptors occurs in the α subunit and the extracellular portion of the ß subunit and in the carboxy terminal domain (approximately 45% identity). Their α subunits reside exclusively on the extracellular face of the plasma membrane and contain an N-terminal cysteine rich domain thought to be involved in the high affinity ligand binding activity displayed by these receptors (387,388). The α subunits are generally thought to be disulfide-linked to each other through what has been defined as class I disulfide bonds (389) Which are highly susceptible to reduction. The α subunits are also anchored to the plasma membrane by disulfide linkages to the extracellular portion of the transmembrane ß subunits by class II disulfide bonds which are resistant to reduction. The intracellular region of ß subunits contain the ATP binding domain (390), tyrosine-specific autophosphorylation acceptor sites (391), the intrinsic protein kinase activity of the receptor (392-396) and serine and threonine phospho-acceptor sites for other endogenous protein kinases (397,398).

Structural properties of the receptor kinases

These receptors possess intrinsic tyrosine-specific protein kinase activity which enables them to undergo autophosphorylation as well as to phosphorylate exogenous substrates. ß subunit autophosphorylation of both receptors is ligand dependent (399,400). Significant work has been carried out on insulin receptor. It is reasonable to assume that a

similar situation exists in the case of the Type I receptor. Autophosphorylation occurs predominantly at tyrosine residues 1146,1151,1152,1316 and 1322 (401,402). The precise order of autophosphorylation and correlation of these autophosphorylation sites with protein kinase activation has not been clearly established to date. It has been suggested that autophosphorylation is a highly concerted reaction in which each phosphotyrosine residue contributes proportionately to kinase activation (403).

In the purified state, the ligand-induced activation leads to autophosphorylation on tyrosine residues due to an intramolecular autophosphorylation reaction. However, ligand stimulation of both receptors in vivo results in rapid tyrosine autophosphorylation (< 1 min) followed by a slower (> 10 min) increase in phosphoserine and phosphothreonine content (392,398,404,405). The increase in phosphoserine/threonine results from either an insulin-dependent activation of intracellular serine/threonine specific protein kinases and/or inhibition of serine/threonine specific protein phosphatases. The functional significance of the insulin-dependent serine/threonine phosphorylation of the insulin receptor has not yet been established.

Transmembrane Signaling

The isolated αß heterodimeric, insulin receptor complex is devoid of kinase activity. Insulin stimulated protein kinase

activity requires the $\alpha_2\beta_2$ heterotetrameric form of the receptor (406). The insulin receptor has been shown to undergo aggregation (407) but whether this is necessary for insulin responsiveness is unclear. Although not studied in as much detail as the insulin receptor, the Type I receptor also apparently requires that αß hetrodimeric subunits associate to yield $\alpha_2\beta_2$ forms in order to display IGF-I dependent protein kinase activation (408). As with insulin Type I receptors appear to undergo receptor aggregation which may be important in transmembrane signaling (409).

Phosphorylation and Biological Responses to the IGFs

To what extent activation of tyrosine kinase activity or phosphoprotein induction is important for the translation of IGF binding into biological responses is not known. Studies in which antibodies to the tyrosine kinase region of the insulin receptor were injected into intact cells suggest that tyrosine kinase activity may not be necessary for transduction of insulin's signal into all it biological responses (410). Some studies in which the tyrosine kinase portion of the insulin receptor was mutated clearly demonstrate that some functions survive (411,412). Although the Type I and insulin receptor's inherent kinase activity may not be the mediator of all responses to ligand, the receptor kinase activity is an important part of the mechanism in eliciting many specific actions (410,411) such as glucose uptake and glycogen synthesis. Several potential in vivo substrate proteins for the

insulin and Type I receptor kinases have been suggested. Insulin stimulated phosphorylation of 95 KDa and 185 KDa protein whereas Type I stimulated phosphorylation of 95,105, and 185 KDa proteins (408) in neuroblastoma cells. The significance of these substrates in insulin and IGF-I action is currently being intensively explored.

Different Species of IGF-I Receptor

Using affinity cross-linking techniques, a subtype of the Type I receptor on rat and human brain membranes was demonstrated (413). This receptor displays a smaller α subunit and has been identified as the neuronal Type I receptor (414). The functional significance of this variant receptor in neurons may relate to the CNS predominance of the truncated IGF-I peptide (415).

(ii)**IGF-II/mannose 6 phosphate receptor**

The IGF-II receptor or type II IGF receptor binds IGF-II with higher affinity than IGF-I (416) and does not recognize insulin (417). The Type II receptor is a single 250 KDa glycoprotein which lacks tyrosine kinase activity and is identical to the cation independent mannose 6-phosphate receptor (418,419,420) which is one of the two receptors which targets lysosomal enzymes to lysosomes.

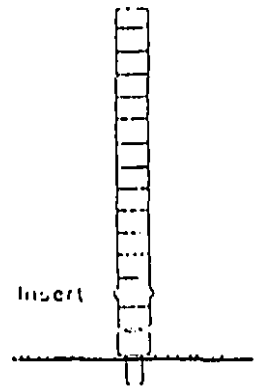
Structure

The primary structure of the human Type II receptor has been deduced from its cDNA sequence obtained from the human hepatoma cell line HEP G2 (418). The structural characteristics are depicted in Figure 6. The Type II receptor consists of a large extracellular domain that is 93% of the receptor protein. There are 19 N-linked glycosylation sites in the extracellular domain. It consists of highly conserved pattern of 8 cysteine residues. There is one major hydrophobic segment of 23 residues which represents the transmembrane domain of the receptor. The cytoplasmic domain is hydrophilic and includes several potential phosphorylation sites on tyrosine, threonine and serine residues. There is no homology with known protein kinases within the cytoplasmic domain.

The human Type II receptor was found to be 99.4% homologous with the human mannose 6-phosphate receptor (420) and 80% with the bovine mannose 6-phosphate receptor (419). The rat Type II receptor was found to be 79% homologous to the bovine mannose 6-phosphate receptor (421). These results along with biochemical and immunochemical evidence suggest that Type II and cation independent mannose 6-phosphate receptors are identical (422,423). The mannose 6-phosphate receptor is located on both the cell surface and in the Golgi apparatus where it binds lysosomal proteins and mediates their transport to the lysosomes (424). IGF-II and mannose 6-phosphate appear to have distinct binding sites on the receptor (423,425) that exhibit cooperativity (421,422). Thus mannose 6-phosphate

Figure 6

Mannose-6-Phosphate/IGF-II receptor



M6P/IGF-II
Receptor

Schematic representation of M6P/IGF-II receptor that binds IGF-II and M6P. The boxes represent repeat sequences. Taken from reference 418.

increases the affinity of the receptor for IGF-II (421,422). However it is not yet possible to explain the physiological relationship between IGF-II and mannose 6-phosphate.

Soluble and truncated M6P/IGF-II receptor

Soluble Type II receptors are found in rat (426), monkey (427) and human (428) plasma and constitute essentially the extracellular domain of the receptor (429). In the rat concentrations are very high in fetal and early neonatal life, and rapidly decrease to much lower levels by 20 days of age, a fall that parallels the tissue abundance of receptor (426). As the M6P/IGF-II receptor is not degraded in the lysosomes, this surface shedding may be a mechanism for receptor clearance. There is a constitutive and rapid movement of the M6P/IGF-II receptor to and from the cell surface which is independent of the binding of ligands (430).

Phosphorylation

The Type II receptor has no intrinsic tyrosine kinase activity but has been reported to be phosphorylated in intact cells (431). The cytoplasmic domain of the receptor contains several potential serine, threonine and tyrosine phosphorylation sites (418,421). Phosphorylation of the receptor in canine kidney membranes on serine and threonine residues which appears to be mediated via activation of phospholipase C (432).

Other structural features suggest potential substrate sites

for protein kinase C and protein kinase A as well as casein kinase(s) (421). Intracellular signaling by the Type-II receptor may be linked to a pertussis toxin-sensitive G protein (433). Type-II receptor is not a classical mitogen receptor, but IGF-II may act as a mitogen via the Type I receptor or the insulin receptor (434).

(b) **Distributions**

IGF receptors are widely distributed in various tissues and cell types. The distribution of Type-I and Type-II receptors is summarized in Table 12 (436).

(c) **Regulation**

(i) **Developmental**

The abundance of the M6P/IGF-II receptor appears to be developmentally regulated, being much higher during the fetal and neonatal period (437). In the rat, the receptor is much more abundant in the neonate than in the adult (438) and it has been suggested that IGF-II may be a growth factor during fetal and neonatal life.

(ii) **Hormonal**

Type-I receptors are regulated according to receptor occupancy, being down regulated by both IGFs as well as insulin according to their ability to bind (439). The number of Type-I receptors appears to be modulated by nutrition (440). Indeed,

Table 12

Tissue distribution of Type-I and Type-II/MSA receptors

Tissue	IGF-I	IGF-II/MSA
Cartilage/bone		
Chondrocytes (bovine)	+	
Chondrosarcoma (rat)		+
Osteocyte (rat)	+	
Placenta		
Human	+	+
Rat		+
Liver		
Human		+
Rat		+
Liver cells		
BRL-3A (rat)	+	+
H-35 hepatoma (rat)		+
Adipocytes		
Rat		+
Fibroblasts		
Human	+	+
Rat	+	+
Chinese hamster	+	
Chick embryo	+	
Fibrosarcoma (human)		+
Endothelium		
Human		+
Bovine		+
Muscle		
Soleus (rat)	+	+
BC 3H-1 (mouse)	+	+
Cental Nervous system		
Brain (human)	+	+
Brain (rat)	+	+
Hypothalamus (human)	+	+
Hypothalamus (rat)	+	+
Pituitary (human)	+	+
Pituitary (rat)	+	+

Table 12 (CONT'D)

Tissue	IGF-I	IGF-II/MSA
GH_3, GH, GC (rat tumor)	+	+
Blood		
Erythrocytes (human)	+	+
IM9 lymphocytes (human)	+	+
T lymphocytes (human)	+	
Brain (human)	+	+
Sertoli cells (rat)	+	+
Pancreatic acini (mouse)	+	+
Kidney (rat)	+	
Human Fetus (10-17 weeks of gestation)		
Brain	+	+
Liver	+	+
Kidney	+	+
Lung	+	+
Adrenals	+	+

Table 12

Type-I and Type-II/MSA receptors are detected in various human and rat tissues and in different cell types in culture. Taken from reference 436.

the expression of these receptors seems to follow the growth state, being expressed in parallel to both physiological and pathophysiological growth (441). In contrast, the Type-II receptor does not appear to be down-regulated. However, insulin increases the number of Type-II receptors on the cell surface (442). The Type-II receptor is rapidly internalized and recycled (443). It has been suggested that insulin induces recycling of the receptor (444, 445). GH increases the number of Type-II receptors as well as modulates the action of insulin on Type-II receptors in adipocytes (446).

(d) **Function**

In the majority of cells, the growth promoting actions of the IGFs or insulin are mediated via the Type-I receptor (447). There are however, specific exceptions to this. IGF-II stimulates growth through the Type-II receptor in a human erythroleukemia cell line (448). Insulin has also been reported to have mitogenic activity through its own receptor in rat hepatoma cells (449), as well as teratocarcinoma cells (450). IGF-I receptor antibodies inhibit the growth promoting actions of either insulin or IGF-II (451). Similarly Type-II receptor antibodies fail to inhibit the growth promoting action of IGF-II (452). Antibody to the Type-II receptor stimulated glycogen synthesis in hepatoma cells. Calcium influx to fibroblasts also appears to be mediated via the Type-II receptor (453). Similarly the acute metabolic actions of IGFs are not always mediated by activation of the insulin receptor, as occurs in

rat adipose cells (454). In human skin fibroblasts, for example, the stimulatory effect of IGFs on glucose transport and metabolism cannot be accounted for by cross-reaction with the insulin receptor (431).

V BIOLOGICAL ACTIONS OF IGFs

The two main biological actions of the IGFs may be summarized as:

1. An insulin-like metabolic action
2. A growth promoting action

The effects of IGFs depend on their mode of administration, with intravenous bolus injections causing acute insulin-like effects and with long-term subcutaneous administration inducing growth (455). This is because of a difference in the availability of the IGFs to the target cells. After a bolus injection, free peptide in excess of that associating with the binding protein induces insulin-like effects. This is supported by the finding that the truncated IGF-I that displays weak binding to the 25 KDa IGFBP elicits a greater insulin-like metabolic response in rats compared with intact IGF-I (456). After long-term subcutaneous administration, IGF associates with the IGFBPs and the animal is thereby protected against an acute hypoglycemic effect. Dissociation of IGF from the IGFBP presumably will depend on the relative affinities of IGF binding to the cell receptor and the IGFBP.

A. **Insulin-like metabolic action**

(a) **In vivo**

The acute metabolic action of IGF-I and IGF-II were reported in both normal and hypophysectomized rats (457,458). An intravenous bolus of either IGF-I or IGF-II induced hypoglycemia and enhanced glucose uptake from serum, glycogen synthesis in muscle, and lipogenesis in adipose tissue (459). IGF-I was more potent than IGF-II. Only modest stimulation of lipid synthesis was observed in normal rats. IGF-I also stimulated amino acid transport. The hypoglycemic effect of IGF-I has been demonstrated in miniature pigs (460) and in healthy adults (461). On a molar basis, IGF-I is far less potent than insulin in inducing hypoglycemia and inhibiting lypolysis; however the levels of epinephrine, norepinephrine, GH, glucagon and cortisol increased similarly after either hormone infusion.

(b) **In vitro**

(i) **Soleus muscle of mice**

IGFs stimulate glucose transport, glycolysis (as demonstrated by increased lactate formation) glycogen and protein synthesis. IGF is 5-10% as potent as insulin on mouse soleus muscle (462).

(ii) **Perfused rat heart**

IGF mimicks all the effects of insulin with a potency about 25% that of insulin (463). Glucose transport and lactate

formation are stimulated at low IGF concentration. At much higher concentration, the contractility of the rat heart is increased by IGF as well as by insulin (464). It appears that IGF stimulates glucose uptake and glycolysis in the rat heart via the IGF receptor, whereas half maximum occupation of the insulin receptor is required for stimulation of contractility (464).

The effects of IGF on muscle in vivo subside 30-40 min after the intravenous injection of the hormone though the half-life of injected unlabeled IGF in the normal rat is 3-4 h. IGF also has short lasting effects on other insulin target tissues. The insulin-like effects of IGF after intravenous injection are seen only with large amounts of IGF that exceed the binding capacity of the carrier proteins. The IGF that is not bound crosses the blood barrier into the interstitial fluid and thereby gains excess to target cells. Available evidence suggests that free IGF has a much shorter half life than IGF bound to the small and large mol. wt. binding proteins. Dissociation of IGF from binding proteins is likely to occur, but the concentrations of free IGF reached in the interstitial fluid appear to be small. Hence no insulin-like effects are elicited. IGF-II is considerably less hypoglycemic than IGF-I (465).

(iii) **Liver cells**

It has been demonstrated that chick embryo liver cells

respond to IGFs in the same way as to insulin (466). IGFs stimulate the synthesis of RNA, protein and glycogen.

B. GROWTH-PROMOTING EFFECTS OF IGFs

(a) In vivo

A correlation between growth and serum IGF-I levels has been noted in humans and animals. In patients with acromegaly, IGF-I levels are 3 fold higher than in normal subjects (467,468). Pituitary dwarfs have low levels of IGF-I, which rise into the normal range when they are treated with growth hormone (469). Hypophysectomy results in a marked decrease in IGF-I levels in the rat (470) and dog (471). Rats bearing GH-producing tumors have very high levels of somatomedin C/IGF-I and grow rapidly (472). After excision of the tumor, GH and IGF-I levels fall and the animals stop growing. In all these situations, IGF-I levels parallel the GH concentration. It appears that IGF-I rather than GH is directly related to growth. GH stimulates the serum level of IGF-I which might well mediate the growth promoting effects of GH (469). Controversial results have been obtained where in one study GH was shown to have direct growth promoting effects on cartilage (473) and in another IGF-I was shown to have growth promoting effects on costal cartilage compared to GH (474). IGF-II was less potent than IGF-I (475). It is likely that GH is acting via local production of IGF-I in cartilage, as IGF-I mRNA levels in cartilage were found to be elevated after GH treatment (475).

GH has distinct effects on the form in which IGF circulates

in serum. GH deficient rats and man lack the large M_r IGF-binding protein in serum (476,470). The serum of IGF-infused rats still lacked this binding protein, but serum from GH treated rats showed the appearance of the large M_r binding protein. All the IGF present in the serum of IGF-infused rats circulated in the form of a 50 K complex, whereas in GH-infused rats endogenous IGF circulated mostly in the form of the 150 K complex.

IGF-I stimulated the growth of hypophysectomized rats (475) as well as Snell dwarf mice (477). IGF-II had weak growth promoting activity in hypophysectomized rats (475), but stimulated the growth of Snell dwarf mice (477). Long-term subcutaneous infusion of recombinant IGF-I stimulated the growth of hypophysectomized rats (458), rapidly growing postweaning normal rats (478), neonatal and preweaning rats (479), IDDM rats (480) as well as Snell dwarf mice (477). Because the growth promoting actions of IGF-I in hypophysectomized rats were less potent than those of GH, it is possible that the actions of GH are mediated through the stimulation of locally produced paracrine IGF-I rather than via circulating levels. Again the delivery of IGFs to their target cells from the circulation represents a complex phenomenon, involving not only the IGFs but also their association with IGFBPs, the production of which is also GH dependent.

(b) **In vitro**

(i) **DNA synthesis and cell proliferation**

With use of primary cell cultures, established cell lines, or organ explants, it has been demonstrated that both IGF-I and IGF-II stimulate DNA synthesis and cell proliferation (455,481). Meiotic division in oocytes is also stimulated by the IGFs (482).

(ii) **Progression Factor**

It has been shown that the IGFs act as progression factors to stimulate cells through the DNA synthesis phase of the cell cycle. This mitotic action occurs in conjunction with competence factors, such as PDGF and FGF, which initiate the cell cycle and prime the cells to respond to progression factors (483). Thus the combination of PDGF and IGF has proven to be as potent a growth promoter as serum.

(iii) **Cell Differentiation**

The IGFs have been demonstrated to stimulate the differentiation of myoblasts (484), osteoblasts (485), and adipocytes (486), as well as oligodendrocytes (487). IGF appears to be a differentiating factor for both ovarian and testicular tissue (488,489).

(iv) **Other Biological Fuctions**

Both IGF-I (490) as well as IGF-II (491) induce erythropoiesis, and IGF-I stimulates granulopoiesis (492) as

well as chemotaxis in endothelial and melanoma cells (493). In rat skeletal muscle cells, IGF-I has been shown to stimulate the expression of the oncogene c-fos (494). The IGFs have been reported to stimulate neurite outgrowth (495). A role in neurotransmission has also been suggested (496). Although the IGFs stimulate the release of acetylcholine from brain slices (497) and catecholamines from chromaffin cells (498), this effect does not appear to be mediated by enhanced precursor uptake or neurotransmittor synthesis. Instead, this action may relate to enhanced Ca^{2+} influx, which has been shown to be induced by IGF-I and IGF-II in fibroblasts (499,500). In retinoblastoma cells, IGF-I stimulates the transport of the putative neurotransmitter glycine into the cells (501). Thus the IGFs have a wide variety of biological actions that have been demonstrated in vitro. A comprehensive hypothesis to cover this variety suggests that these peptides have an anabolic action the result of which will be determined by cellular programing at the time the IGFs act on target cells. Whether the response elicited is, for example, proliferation, glycogen synthesis, or enhanced neurotransmittor release must depend on the state of the target cell. If the cells are in the hyperplastic phase, then the IGFs stimulate proliferation. However, if the cells are undergoing hypertrophic growth, or are mature nondividing cells such as neurons, then other anabolic processes, such as protein synthesis, are stimulated.

VI. AIMS OF THE PRESENT STUDY

IGF-I is an important mediator of GH action particularly during postnatal and adolescent growth periods (24). Liver is the major source of production of circulating levels of IGF-I in the blood. The evidence that more than 90 % of IGF-I in blood is produced by liver comes from liver perfusion study in the rat (59) and also from hypophysectomized (34,35) and diabetic rats (61,62). IGF-I mRNA is 200 X more abundant in rat liver than in any other tissues (50).

Previous work on hormonal regulation of hepatic IGF-I mRNA levels in rat in vivo

The circulating IGF-I and hepatic IGF-I mRNA levels are reduced in hypophysectomized (34,35) and diabetic rats (61,62) and reach control levels following GH and insulin treatment respectively. Ovine prolactin has been reported to stimulate hepatic IGF-I mRNA abundance and circulating levels in hypophysectomized rats (129) perhaps by acting via somatogenic receptors. Dexamethasone inhibits circulating levels of IGF-I and hepatic IGF-I mRNA abundance in intact rat and also inhibits GH action on circulating levels of IGF-I and hepatic IGF-I mRNA abundance in hypophysectomized rats (133). Thyroid hormones have relatively little direct effect on stimulation of circulating levels of IGF-I and on hepatic IGF-I mRNA abundance in hypophysectomized rats but potentiate GH induction of

hepatic IGF-I mRNA levels and circulating levels of IGF-I (141). Octreotide, a somatostatin analog has been shown to have no effect on hepatic IGF-I mRNA levels or on the circulating levels of IGF-I in hypophysectomized rats. But it inhibits the stimulatory effect of GH on hepatic IGF-I mRNA levels and on the circulating levels of IGF-I in hypophysectomized rats (149).

Previous work by other investigators on hormonal regulation of IGF-I mRNA levels in cultured rat hepatocytes

Johnson et al (173) showed 2 to 3 fold stimulation of IGF-I mRNA levels by each of GH and insulin. No further stimulation was observed on combining GH and insulin. A similar effect was also observed on IGF-I secretion into the culture medium. Boni-Schnetzler et al (174) also reported a 2 to 3 fold stimulation of IGF-I mRNA levels in rat hepatocytes by each of GH and insulin and an additive effect on IGF-I mRNA stimulation when GH and insulin were combined. In both cases, hepatocytes were used 24 h after plating and the culture medium used by Johnson et al (173) contained T_3, hydrocortisone and insulin, whereas the culture medium used by Boni-Schnetzler et al (174) contained insulin and dexamethasone.

Tollet et al (176) showed no effect of insulin or T_3 on IGF-I mRNA levels but each potentiated the GH effect on IGF-I mRNA levels in cultured rat hepatocytes. The culture medium used in this study contained insulin, and the hepatocytes were

maintained in culture for 66 h before the experiment was initiated.

Since different results have been reported on insulin's effect on IGF-I mRNA levels in hepatocytes, and the evidence for a role of other hormones (direct or modulatory) on the GH effect are lacking, we initiated the present project to investigate the role of various hormones on IGF-I mRNA levels and IGF-I peptide production in rat hepatocytes.

The in vitro primary culture system was chosen as it more closely resembles the natural state of the liver than any established cell line. The procedure for culturing and maintaining rat hepatocytes under serum free conditions was already established in our laboratory (130).

Primary cultured rat hepatocytes maintained under serum free conditions in hormonally defined medium containing insulin, EGF, hydrocortisone, glucagon, T_3, selenium, ornithine, ethanolamine and lactic acid (9F medium) (130) expressed low levels of IGF-I mRNA. The addition of bGH stimulated IGF-I mRNA levels 10 fold. In order to decide which hormones should be removed from the system without affecting the viability of the cells and at the same time observe the stimulatory effect of bGH on IGF-I mRNA levels and IGF-I secretion into the medium, one hormone was removed at a time from the medium and the effect of GH on IGF-I mRNA level and IGF-I peptide production was noted.

When glucagon was removed from the medium, GH's effect on IGF-I mRNA levels and IGF-I peptide production was markedly

reduced, indicating that glucagon was an important modulator of the GH effect on hepatic IGF-I production. Finally the medium containing selenium, ethanolamine, ornithine and lactic acid (4F medium) was used for hepatocyte cultures.

Hepatocytes were plated in a medium containing 10 % fetal calf serum and the medium was changed to 4F medium. The effect of bGH on IGF-I mRNA level was tested after 24, 48 and 72 h of plating the cells, in order to determine the duration of the culture which would optimize the effect of bGH on IGF-I mRNA stimulation. From the above experiment, we observed that maintaing hepatocytes in culture for 72 h in a serum free medium was essential to obtain the maximum response of bGH on IGF-I mRNA stimulation.

We addressed the folowing questions in the current study.

1. Does glucagon stimulate IGF-I mRNA levels and IGF-I secretion in hepatocytes ?

2. Does glucagon have a modulatory effect on GH induction of IGF-I mRNA levels and IGF-I peptide production in hepatocytes?

The results obtained are discussed in Chapter 2. Glucagon was found to have a modest effect on stimulation of IGF-I mRNA levels and IGF-I peptide production but produced a synergistic effect when added in combination with GH on stimulation of IGF-I mRNA levels and produced an additive effect on stimulation of IGF-I peptide production in hepatocytes.

3. We also investigated in vivo inhibitory effect of octreotide on GH stimulation of hepatic IGF-I gene expression

and on circulating levels of IGF-I. As discussed in chapter 3 this effect could have been due to low levels of glucagon in serum when hGH and octreotide were administered in hypophysectomized rats. Octreotide was also found to have a direct inhibitory effect on IGF-I mRNA levels in cultured rat hepatocytes.

The results obtained with glucagon and GH on IGF-I production, led us to pose the following questions which are discussed in Chapters 4 and 5.

4. What other hormones when added in combination with glucagon or bGH produce a synergistic effect on stimulation of IGF-I mRNA levels in hepatocytes ?

5. What pathways are involved in mediating glucagon plus GH effect on IGF-I gene expression ?

6. Since PKA and PKC pathways appeared to be involved in the bGH plus glucagon effect on IGF-I mRNA stimulation, we investigated if the synergistic effect on the stimulation of IGF-I mRNA levels was obtained by elevating PKC and PKA levels.

7. Is new protein synthesis required for glucagon plus bGH synergistic effect on IGF-I mRNA levels ?

8. Do bGH and glucagon act at a transcriptional level to stimulate IGF-I mRNA levels or are their effects at a posttranscriptional level to stabilize IGF-I mRNA ?

INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1

IGFs do not circulate in the body as free peptides but are tightly bound to high affinity IGFBPs. Six IGFBPs have been

cloned to-date (203-211). The functions and regulations of these BPs are not well understood. They have been shown to increase the half life of IGFs (354) and act as reservoirs to release IGFs into the circulation as needed. When IGFs are bound to IGFBPs they are unavailable to cell surface receptors and hence the organism is protected from the hypoglycemic effect of IGFs (356).

Previous in vivo study of other investigators on hepatic IGFBP-1 production

The circulating levels of IGFBP-1 and hepatic IGFBP-1 mRNA are elevated in hypophysectomized (318) and diabetic rats (310) and they reach control levels on treatment with GH (318) and insulin (310) respectively. Controversial results have been reported about the effect of GH on hepatic IGFBP-1 mRNA levels in hypophysectomized rats (310). It has been suggested that the elevation of IGFBP-1 mRNA levels in hypophysectomized rats is not due to GH deficiency but may be due to insulin deficiency (310). Dexamethasone has also been shown to stimulate circulating levels of IGFBP-1 and hepatic IGFBP-1 mRNA levels in rat (325).

Previous in vitro studies of other investigators on IGFBP-1 mRNA levels

Insulin has been shown to inhibit (230,334) whereas dexamethasone stimulates (230,333) IGFBP-1 mRNA levels in H4IIE

rat hepatoma cells. Insulin also inhibits IGFBP-1 mRNA levels in rat hepatocytes (231). The effects of other hormones on IGFBP-1 mRNA levels in hepatocytes or in other cell lines are not known.

The questions addressed in this study

We decided to use cultured rat hepatocytes maintained under serum-free conditions, as described previously, to study the hormonal regulation of hepatic IGFBP-1 mRNA levels.

The following questions were addressed:

1. What are the effects of bGH and glucagon on IGFBPs secreted by hepatocytes in the medium ?

As discussed in Chapter 2, bGH was found to inhibit whereas glucagon was found to stimulate IGFBPs secreted by hepatocytes in the culture medium. Since it was difficult to characterize which BPs were secreted into the medium, we determined if IGFBP-1 mRNA was expressed by hepatocytes in culture. As discussed in Chapter 6, we further investigated the effects of bGH and glucagon on IGFBP-1 mRNA levels, as these were novel observations and have not been reported previously.

The inhibitory effect of bGH and the stimulatory effect of glucagon on IGFBP-1 mRNA levels led us to pose the following questions.

2. What are the pathways involved in bGH and glucagon effect on IGFBP-1 mRNA levels ?

3. Do bGH and glucagon require synthesis of new protein(s) to mediate their effects on IGFBP-1 mRNA levels?

4. What are the effects of insulin, dexamethasone and T_3 on IGFBP-1 mRNA levels ?

5. Are the effects of bGH and glucagon on IGFBP-1 mRNA at the level of gene transcription or at a posttranscriptional level on mRNA stability ?

The results obtained in this study are discussed in Chapter 6.

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CHAPTER 2

THE DIFFERENTIAL REGULATION BY GLUCAGON AND GROWTH HORMONE OF INSULIN-LIKE GROWTH FACTOR (IGF)-I AND IGF BINDING PROTEINS IN CULTURED RAT HEPATOCYTES

PREFACE TO CHAPTER 2

This chapter mainly deals with the effect of bovine growth hormone (bGH), glucagon and bGH plus glucagon on IGF-I secretion, IGF-I mRNA expression and IGFBP(s) production in cultured rat hepatocytes.

Our preliminary results indicated that glucagon appeared to stimulate as well as potentiate GH effect on the stimulation of IGF-I production in cultured rat hepatocytes. We also investigated if this effect was at the mRNA level. We further investigated the effects of bGH, glucagon and their combination on IGF-I production, IGF-I mRNA expression and IGFBP-1 production in hepatocytes. bGH and glucagon individually stimulated IGF-I mRNA levels modestly whereas they produced a synergistic effect and stimulated IGF-I mRNA levels 10 to 12 fold when combined. IGF-I peptide production in the medium was also measured after 24 h of incubation with bGH, glucagon and their combination. A modest stimulation of IGF-I secretion into the medium was observed with bGH and glucagon individually but produced an additive effect on the stimulation of IGF-I secretion into the medium when bGH and glucagon were combined. The effects of bGH, glucagon and bGH plus glucagon was also measured on IGFBP(s) production by hepatocytes in their culture medium. Since the antibodies against individual BPs were not available, it was not possible to identify which BPs were secreted into the medium. From Western ligand blotting, it appeared that IGFBPs 1 and/or 2 and 4 may be produced by

hepatocytes. Glucagon's stimulatory response on IGFBP(s) secretion was observed 12 h after its addition, whereas GH's inhibitory effect on IGFBP(s) production was only observed 24 h after its additon to hepaocytes.

ABSTRACT

The liver is a major site of production of insulin-like growth factor-I (IGF-I) and IGF binding proteins (IGF-BPs). GH decisively influences IGF-I production. To study the role of GH and glucagon in the regulation of IGF-I and IGF-BP production, we examined IGF-I and IGF-BPs secreted by primary rat hepatocytes cultured in a serum-free medium. Glucagon (1×10^{-8} M) stimulated IGF-I secretion and IGF-BP secretion. Bovine GH (bGH, 300 ng/ml) stimulated IGF-I secretion but suppressed IGF-BP secretion. Combining bGH and glucagon significantly augmented IGF-I secretion above the level seen with each individual agent. The inhibitory effect of bGH on IGF-BP secretion was reversed by glucagon. The major species of IGF-BPs secreted by hepatocytes were found, on Western ligand blotting, to be 24K and 30-34K. All species of secreted IGF-BPs appeared to be comparably affected by glucagon, bGH, and their combination.

Northern analysis of IGF-I mRNA revealed three transcripts of 0.7-1.1 kilobases (kb), 1.8 kb and 7.0 kb. Glucagon stimulated IGF-I mRNA levels 1.8- to 2.0-fold, whereas bGH stimulated IGF-I mRNA levels 2.0- to 2.5-fold. When hepatocytes were incubated with glucagon and bGH for 6 h, IGF-I mRNA levels were augmented 10 fold. Glucagon, in the presence of 50 ng/ml bGH, had a dose-dependent effect on IGF-I mRNA accumulation from a 6-fold level of stimulation at 50 ng/ml of glucagon to a 9-fold level of stimulation at 1000 ng/ml glucagon.

This study has demonstrated that glucagon, as well as GH, has significant effects on the production of both IGF-I and IGF-BPs. Of particular interest was the marked augmentation of hepatic IGF-I messenger RNA levels and the reversal of the low levels of IGF-BP production seen on adding glucagon to bGH.

INTRODUCTION

Insulin-like growth factor-I (IGF-I) is thought to be an important mediator of GH action, particularly during the postnatal and adolescent growth periods (1). In additon to the liver, the major source of circulating IGF-I (2), other tissues such as kidney, lung, heart, and testes have been shown to produce IGF-I and to contain IGF-I messenger RNA (mRNA) (3,4). The level of IGF-I mRNA in rat liver and in other tissues has been shown to be GH dependent (5-8). These observations suggest that, in addition to its potential endocrine role, IGF-I functions in an autocrine/paracrine fashion.

IGF-I and -II do not exist in body fluids as free polypeptides, but are complexed to specific IGF-binding proteins (IGF-BPs) (9). Although the role of these IGF-BPs is unclear they do prolong the half-life of IGFs (10). They may act synergistically with IGFs (11-13) or may inhibit their actions (11,12,14,15). Multiple IGF-BPs have been detected in plasma and other biological fluids (11,16,17). Three IGF-BPs have been recently sequenced and their complementary DNAs (cDNAs) cloned: a nonglycosylated 25K BP (IGF-BP-1) (11,18), a nonglycosylated 31K BP (IGF-BP-2) (19,20), and a glycosylated BP of mol wt 50-53K (IGF-BP-3) (21,22). IGF-BP-3 varies directly with GH (23), whereas IGF-BP-1 and IGF-BP-2 appear to increase in GH deficiency (24,25).

Previous studies using cultured hepatocytes have

demonstrated relatively modest effects of GH on hepatocyte levels of IGF-I mRNA (6). We have employed hepatocytes maintained in a serum free medium to explore the regulatory role of GH on the hepatic production of IGF-I and IGF-BPs and on IGF-I mRNA levels. In the course of examining the role of other hormones we identified a previously unrecognized role for glucagon in augmention especially in conjunction with GH, the expression of hepatocyte IGF-I mRNA and secretion of IGF-I and IGF-BPs by hepatocytes.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats weighing 180-200g were obtained from Charles River Canada Inc. (St. Constant, Quebec, Canada). The rats were housed under standard lighting conditions (light on from 0700-2000h) and maintained on Purina Chow (Ralston-Purina, St. Louis, MO).

Hormones and chemicals

Bovine GH (USDA-bGH-B1, 1.4 IU/mg) was kindly supplied by the USDA Animal Hormone Program (Beltsville, MD). IGF-I antiserum (UKB 487) was kindly provided through the Hormone Distribution Program of the NIDDK (Bethesda, MD), courtesy of Drs. J.J. Van Wyk and L Underwood. IGF-I was a generous gift from Ciba-Geigy Ltd. (Basel, Switzerland) and Chiron Corp. (Emeryville, CA). Ornithine, L-lactic acid, glucagon, selenium, ethanolamine, EGTA, protamine sulfate, Denhardt's solution (50X), salmon sperm DNA, and activated charcoal (untreated powder) 100-400 mesh were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase was from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12 Nutrient mixture, and fetal calf serum (FCS) were from GIBCO (Grand Island, NY). Tissue culture plates (100 mm), penicillin, streptomycin and Fungizone were from Flow Laboratories (Rockville, MD). Collagen (Vitrogen 100) was from

Collagen Corporation (Palo Alto, CA). Carrier free ^{125}I was purchased from New England Nuclear Corp. (Boston, MA). Formamide, guanidinium isothiocyanate, and Cesium chloride were purchased from Amnicon (Danvers, MA).

Preparation of primary hepatocyte cultures

Hepatocytes were isolated from male Sprague Dawley rats weighing 180-200 g by perfusion in <u>in situ</u> with collagenase as described previously (26,27). At the end of perfusion, the liver was removed and placed in DMEM/F12 medium containing 10 mM HEPES and 20 mM $NaHCO_3$ supplemented with 10% fetal bovine serum, 500 IU/ml penicillin, 500 ug/ml streptomycin, and 1.25 ug/ml Fungizone.

An aliquot of cell suspension was diluted 5:1 with Trypan blue to determine cell viability, which was 80% at the onset of culture. Cells were plated at a density of 5 X 10^6 cells/100 mm culture plates. The plates were previously coated with a 10% solution of collagen and incubated at 37 C in a humidified atmosphere (5% CO_2). After overnight attachment, the culture medium was changed to a serum-free medium supplemented with 0.4 mM ornithine, 2.25 ug/ml L-lactic acid, 2.5 X 10^{-8} M selenium, and 1 X 10^{-8} M ethanolamine (27). Thereafter the media were changed daily throughout the duration of the experiment.

Hormonal stimulation and media collection

After 72 h fresh medium with or without bGH, glucagon, or

bGH and glucagon was added to the cells. The bGH and glucagon concentrations were 300 ng/ml and 500 ng/ml, respectively in all the experiments except where indicated otherwise. Media were collected at 3 h, 6 h, 12 h, and 24 h after the addition of hormones. The media were centrifuged at 1000 rpm for 10 min to remove cell debris and were frozen at -20 C for the later determination of IGF-I and IGF-BPs.

Preparation of RNA

Total RNA from freshly isolated hepatocytes and cultured rat hepatocytes was prepared by the guanidinium isothiocyanate-Cesium chloride technique (28). Freshly isolated hepatocytes were homogenized in 5 vol 4 M guanidinium isothiocyanate buffer containing 0.1 M Tri-Cl (pH 7.5) and 1% ß-mercaptoethanol. Hepatocyte monolayers were lysed in the above buffer (2 ml/100 mm culture plate). The homogenates or cell lysates were layered on 4 ml 5.7 M Cesium chloride containing 0.01 M EDTA (pH 7.5) in 13 ml Beckman Ultra Clear centrifuge tubes. The samples were centrifuged at 33,000 rpm in a Beckman Ultracentrifuge (model LM8) using an SW 40 rotor for 22 h at 20 C. The RNA pellet was dissolved in 0.3 M sodium acetate buffer pH 5.2 and precipitated with 2.2 vol ethanol at -20 C overnight. The pellet was dried in a speedvac and stored at -70 C.

Northern blot analysis

Total RNA samples (40 ug/ml) were denatured in 50% formamide and 2.2 M formaldehyde at 65 C for 15 Min. The samples were

electrophoresed through 1.5% agarose, 2.2 M formaldehyde denaturing gel using 20 mM 3-(N-Morpholino) propanesulfonic acid buffer containing 8 mM sodium acetate and 1 mM EDTA (pH 8.0) (29). Ribosomal RNAs (28S and 18S) were visualized under UV light to ensure the integrity of RNA samples. RNA was transfered to Hybond-N membranes by capillary transfer using 1 M sodium chloride. RNA was fixed to the membrane by baking at 80 C for 2 h. One lane containing RNA molecular size markers was removed from the rest of the membrane and stained with 0.04% methylene blue (29).

IGF-I cDNA probe

A rat IGF-I cDNA consisting of a 422 basepair fragment, coding for the pre-, B, C, D, and part of the E domains of IGF-I peptide, inserted in plasmid PUC 19, and transfected in Escherichia coli was kindly provided by Drs. Roberts and D. LeRoith of the NIH. The IGF-I cDNA insert was isolated by ECoR I restriction enzyme digestion, followed by electrophoresis in low melting point agarose. IGF-I cDNA (20 ng) was labeled with [^{32}P]dCTP by random primer labeling technique (30) using the procedure described in the BRL random primer labeling system. The probe was labeled to a specific activity of 10^9 dpm/ug DNA.

Hybridization

Membranes were prehybridized in 5 X SSPE (1 X SSPE = 180 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA pH 7.4), 50% formamide, 5 X

Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 100 ug/ml salmon sperm DNA for 2 h at 42 C. The prehybridization solution was replaced by a fresh hybridization solution containing the labeled probe at a final concentration of 1 X 10^6 cpm/ml, and the incubation was continued for 16-18 h at 42 C. The blots were washed twice at room temperature in 2 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 0.1% SDS for 30 min each and once at 60 C in 1 X SSC, 0.1% SDS for 15 min. The blots were exposed to Kodak X-AR film (Eastman Kodak Co., Rochester, NY) at -70 C for 2-3 days.

Iodination of IGF-I

IGF-I was labeled with $Na^{125}I$ by a chloramin T procedure as previously described (31), to a specific activity of 130 uCi/ug.

IGF-I RIA

Binding proteins and IGF-I in conditioned media were resolved by acid (0.01 N HCl) gel chromatography on Sephadex G-75. Medium (10 ml) was lyophilized and reconstituted in 1.0 ml 0.01 N HCl, and an aliquot of 800 ul was applied to Sephadex G-75 column (1.5 X 50 cm) calibrated with blue dextran and [^{125}I]IGF-I before sample application. The column fractions were assayed for IGF-I using antiserum (UBK 487) as previously described (31).

Charcoal binding assay for IGF-BPs

The amount of IGF-BP present in conditioned media was determined by a charcoal binding assay using protamine sulfate (32,33). Labeled [^{125}I]IGF-I (10,000 cpm) and different volumes of media were added to 50 mM Tris-HCl, 0.5% BSA (ph 7.4) at a final vol of 0.5 ml. After overnight incubation at 4 C, 1 ml 1% activated charcoal and 0.2 ug/ml protamine sulfate were added to each tube. The tubes were centrifuged at 3,000 rpm at 4 C for 30 min and the pellets counted to determine free [^{125}I]IGF-I.

Western ligand blotting

Media samples were concentrated 10 fold using Centricon-10 microconcentrators (10,000 mol wt cut off) and subjected to SDS-PAGE using 12% acrylamide as described elsewhere (34). Separated proteins were electroblotted onto nitrocellulose filters (0.45 uM pore size) using a Bio-Rad transblot unit. Filters were blocked and incubated with [^{125}I]IGF-I (4 X 10^5 cpm) per 3 ml saline containing 1% BSA and 0.1% Tween overnight at 4 C and visualized by autoradiography (32,35).

RESULTS

Secretion of IGF-I in the culture medium

In order to study the hormonal regulation of IGF-I secretion by cultured rat hepatocytes, the basal level of IGF-I secretion was reduced by using medium containing nutritional factors only as described in Materials and Methods. The basal level of IGF-I secretion under these conditions was one fourth that observed with complete medium (27).

In order to quantitate IGF-I production by hepatocytes when incubated in the absence or presence of bGH and/or glucagon for 24 h, we subjected culture medium to acid-gel chromatography to separate IGF-BPs from IGF-I. When binding proteins were separated from IGF-I, the latter were easily and reliably measured by RIA. As shown in Fig. 1, a considerable amount of IGF-I was secreted into the medium (8 ng/ml/10^6 cells) when cells were maintained in medium containing nutritional factors only. A marked stimulation of IGF-I secretion into the medium was observed with glucagon alone (total secretion was 22.8 ng/ml/10^6 cells) as well as with bGH alone (total secretion was 22.7 ng/ml/10^6 cells). Combining bGH and glucagon resulted in an additive effect on IGF-I secretion (total secretion was 40.6 ng/ml/10^6 cells). Table 1 summarizes these results and those of a second experiment showing the same essential features as the first.

Figure 1. Effect of bGH and/or glucagon on IGF-I secretion by cultured hepatocytes after 24 h of incubation

Acid gel chromatogram of conditioned media from hepatocytes treated without or with bGH and/or glucagon for 24 h. Hepatocyte cultures were prepared as described in Materials amd Methods. Cells were exposed to bGH and/or glucagon 72 h after plating, and media samples were collected 24 h after initiating hormonal treatment. Media samples were concentrated 10 fold and subjected to acid gel chromatography using a Sephadex G-75 column equilibrated with 0.01 N HCl to separate IGF-I from IGF-BPs. Samples were eluted using 0.01 N HCl. IGF-I in every second column fraction was determined by RIA. A second study showed similar results.

Figure 1

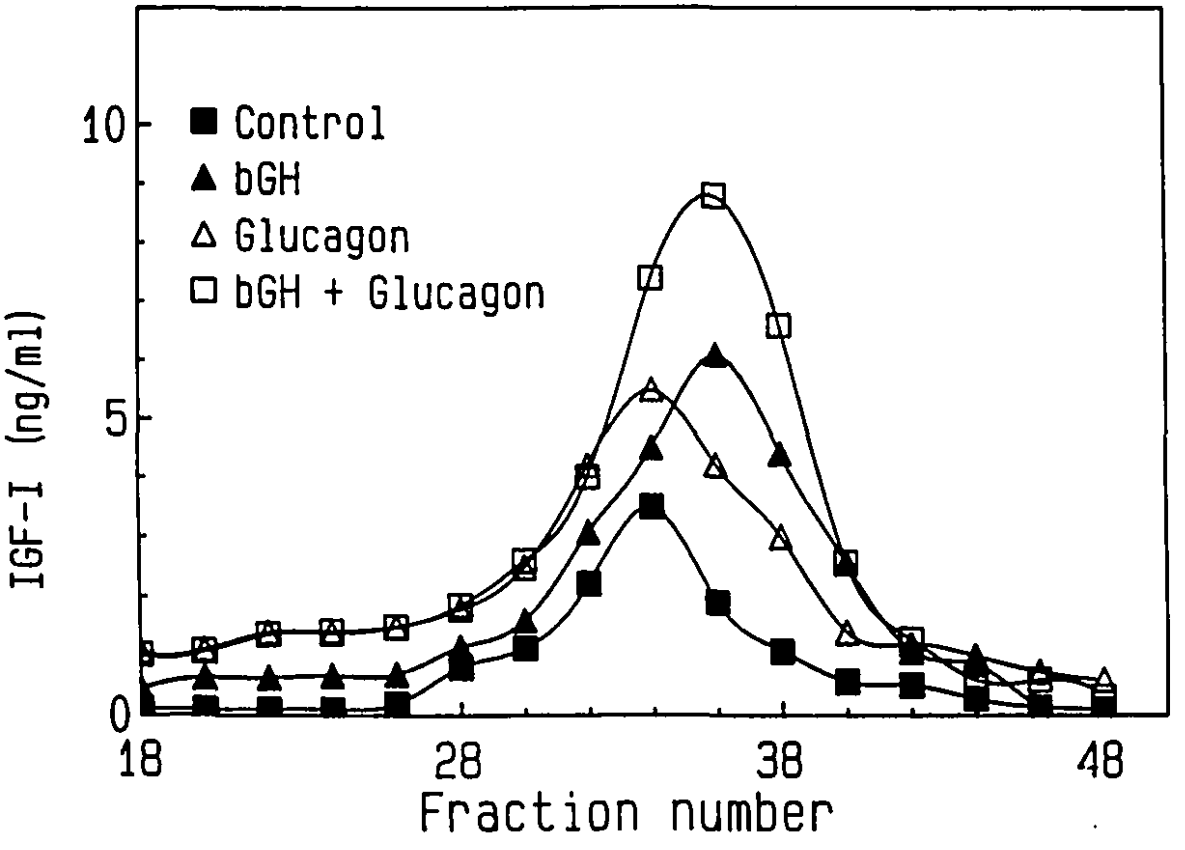

Control
bGH
Glucagon
bGH + Glucagon
IGF-I (ng/ml)
10
5
0
18
28
38
48
Fraction number

Table 1. Effect of bGH and/or glucagon on IGF-I secretion by cultured rat hepatocytes

Treatment	IGF-I secreted ($ng/10^6$ cells/24h)	
	Exp. 1	Exp. 2
Control	8.0	9.84
bGH	22.7	14.73
Glucagon	22.8	15.98
bGH + glucagon	40.6	28.95

Hepatocytes were treated with bGH and/or glucagon for 24 h. At the end of the incubation period, conditioned media were collected and assayed for IGF-I as described in Materials and Methods. Total nanograms of IGF-I secreted per 10^6 cells were calculated by adding the amount of IGF-I in each column fraction and expressing that per 10^6 cells.

Time course of bGH and/or glucagon effects on IGF-I mRNA expression

GH has been shown to stimulate IGF-I mRNA expression in hepatocytes (6) and in hypophysectomized rat liver (5). To address the question whether the effects of glucagon and/or bGH observed on IGF-I secretion (Fig. 1) were at the RNA level, a study of IGF-I mRNA expression was carried out by Northern analysis for the time periods indicated in Fig 2A. Northern blots of total RNA isolated from hepatocyte cultures were probed with ^{32}P-labeled IGF-I cDNA and revealed multiple IGF-I RNA transcripts of approximately 0.7-1.1 kilobases (kb), 1.8 kb, and 7.0 kb as described previously (4,5,8). Increased accumulation of all IGF-I RNA transcripts was evident after 3 h of glucagon and bGH additon, reached maximum accumulation by 6 h, and declined by 24 h but remained above control levels.

Quantification of IGF-I mRNA transcripts by densitometric scanning

Northern blots of IGF-I mRNA (Fig. 2A) were quantitated by scanning the 0.8-1.1 kb and 7.0 kb transcripts. The data in Fig 2B indicate that, after 6 h of incubation, bGH alone stimulated IGF-I mRNA accumulation about 2- to 2.5-fold, whereas glucagon stimulated IGF-I mRNA accumulation 1.8- to 2.0 fold. Combining bGH and glucagon resulted in 10-fold stimulation of IGF-I mRNA accumulation. This stimulatory effect was evident at 3 h of incubation and declined by 24 h.

Figure 2. Time dependent effect of bGH and/or glucagon on IGF-I mRNA accumulation in cultured hepatocytes

2 A. Autoradiogram of Northern blots of total RNA from hepatocytes hybridized with IGF-I cDNA probe. Total RNA samples (40 ug) prepared from hepatocytes treated with no hormones (lane 1), with bGH (lane 2), with glucagon (lane 3), or with a combination of bGH and glucagon (lane 4) for the time periods indicated were subjected to electrophoresis on 1.5% agarose 2.2 M formaldehyde gels and transfered to Hybond-N membranes. Hybridization was carried out with a ^{32}P-labeled IGF-I cDNA probe as described in Materials and Methods. This experiment was carried out on two different occasions, and identical results were obtained.

Figure 2

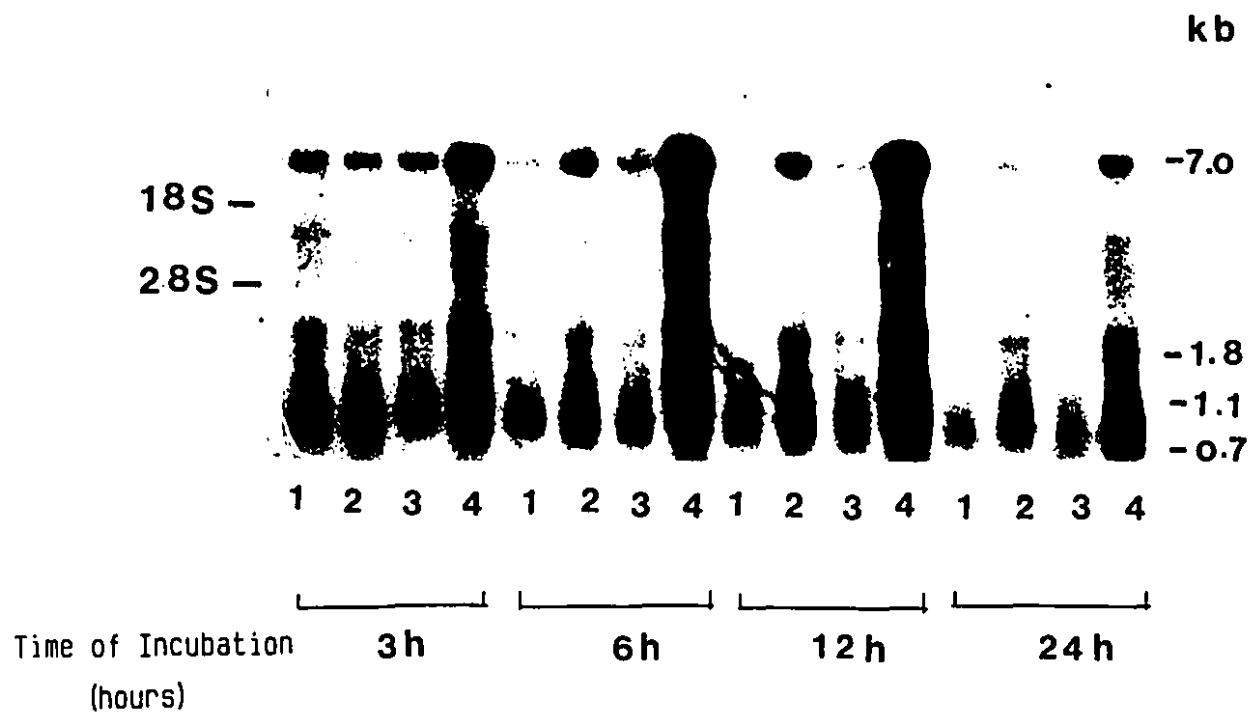
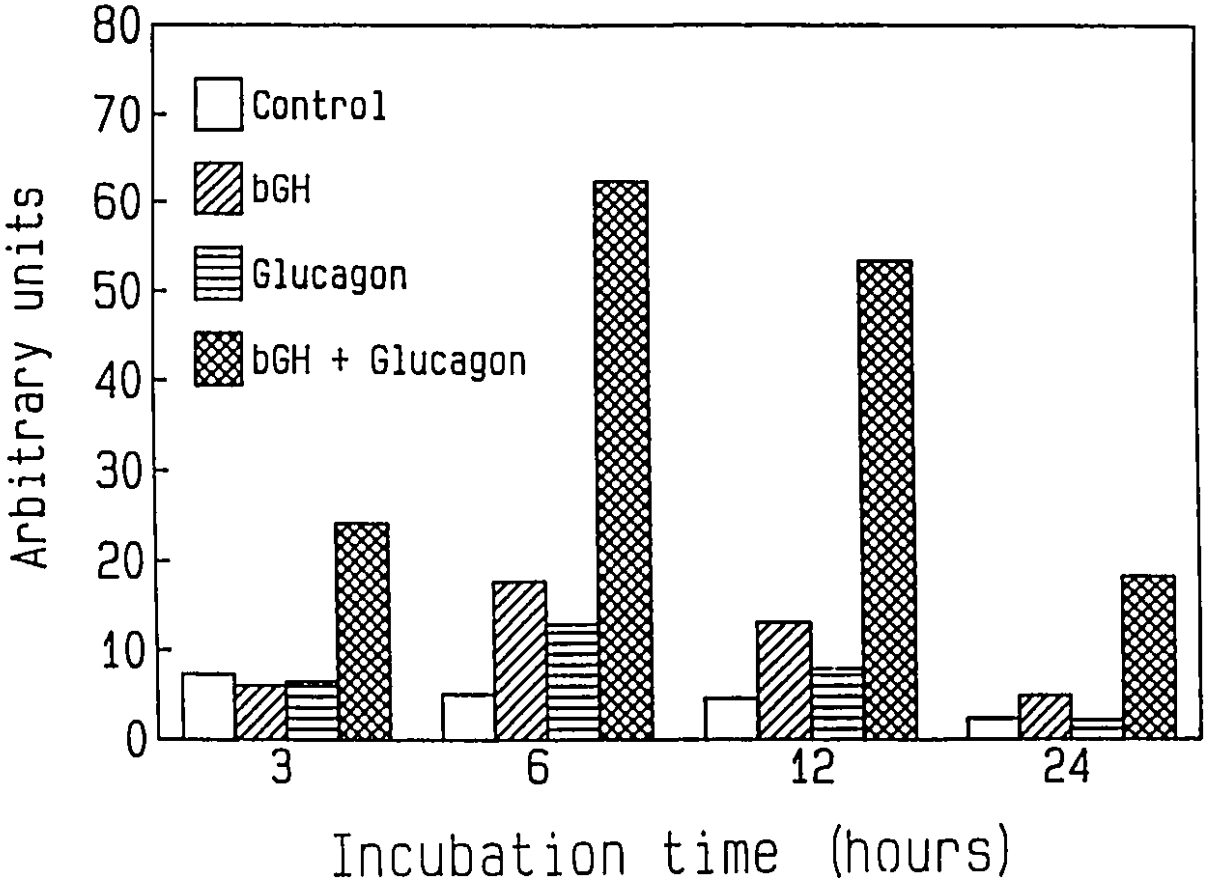

kb
18S
28S
-7.0
-1.8
-1.1
-0.7
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Time of Incubation (hours)
3h
6h
12h
24h

Figure 2

2 B. IGF-I mRNA levels in Northern blots (2A) were quantitated by densitometric scanning of 0.7-1.1 kb and 7.0 kb transcripts, and their values are expressed as arbitrary units.

Figure 2 B


80
70
60
50
40
30
20
10
0
Arbitrary units
Control
bGH
Glucagon
bGH + Glucagon
3
6
12
24
Incubation time (hours)

Dose response of bGH and/or glucagon effects on IGF-I mRNA

A dose-response study of the bGH effect on IGF-I mRNA after 6 h of incubation revealed a 2-fold stimulation of IGF-I mRNA accumulation at 10 ng/ml bGH. Thereafter increasing the concentration of bGH from 50-500 ng/ml showed no further stimulation of IGF-I mRNA accumulation (Table 2). Glucagon produced a dose-dependent stimulation of IGF-I mRNA accumulation, as the glucagon concentrations were varied from 10-500 ng/ml. Maximum stimulation (2-fold) was observed with 500 ng/ml glucagon after 6 h of incubation (Table 2).

Since a synergistic effect of IGF-I mRNA accumulation was observed at supraphysiological doses of bGH (300 ng/ml) and glucagon (500 ng/ml), a dose-response study of glucagon's effect on IGF-I mRNA accumulation in the presence of 50 ng/ml bGH, was performed. The data presented in Fig. 3 reveal a dose-dependent synergistic effect of glucagon on IGF-I mRNA accumulation.

IGF-I transcripts (0.7-1.1 kb and 7.0 kb) were quantitated by densitometric scanning, and the data are presented in Fig. 3B. The abundance of IGF-I mRNA levels (sum of the levels of the major transcripts) in different samples was expressed relative to the IGF-I mRNA levels seen in freshly isolated hepatocytes. A marked decrease in the level of hepatocyte IGF-I mRNA was evident after 3 days of culture in serum-free medium containing only nutritional factors. In the presenece of bGH (50 ng/ml), glucagon, at 50 ng/ml, stimulated IGF-I mRNA 6-fold and 1000 ng/ml stimulated IGF-I mRNA 9-fold. Since the maximum

Table 2. The dose-dependent effect of bGH or glucagon treatment of cultured rat hepatocytes on IGF-I mRNA levels

Concentration of hormone (ng/ml)	IGF-I mRNA levels relative to control	
	bGh treated	Glucagon treated
0	1.0	1.0
10	2.1	1.1
50	1.6	1.3
100	1.7	1.4
300	1.8	1.6
500	2.0	1.7

Hepatocytes were treated for 6 h with no (control) or different concentrations of bGH or glucagon. At the end of this incubation period, total RNA was prepared from the cells, and Northern blot analyses were performed as described in Materials and Methods. IGF-I mRNA was quantitated by densitometric scanning of the transcripts (0.7-1.1 kb and 7.0 kb). Levels are expressed relative to that in control hepatocytes, which is normalized to 1.0.

Figure 3. Dose-dependent effect of glucagon/ bGH-stimulated IGF-I mRNA levels in cultured hepatocytes

3 A. Autoradiogram of Northern blots of total RNA prepared from freshly isolated hepatocytes (FH) and cultured hepatocytes. Total RNA samples (40ug) from hepatocytes treated with various concentrations of glucagon in the presence of 50 ng/ml bGH for 6 h were electrophoresed in 1.5% agaros-2.2 M formaldehyde gels and transfered to Hybond-N membranes. Hybridization was carried out with a ^{32}P-labeled IGF-I cDNA probe as described in Materials and Methods.

Figure 3 A

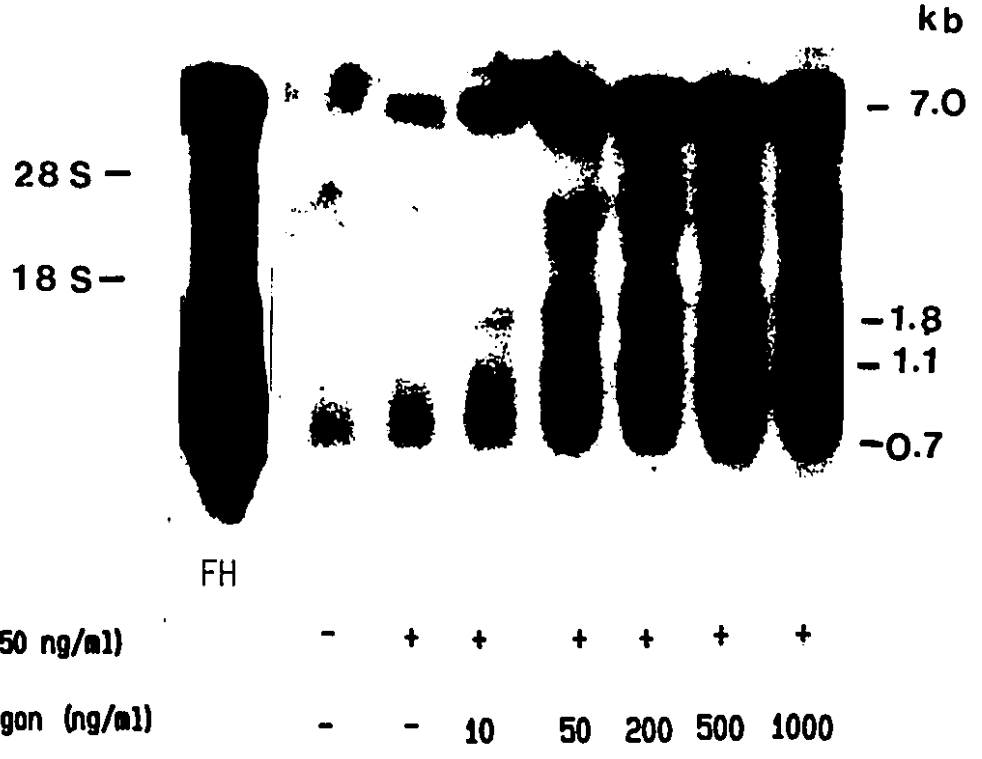
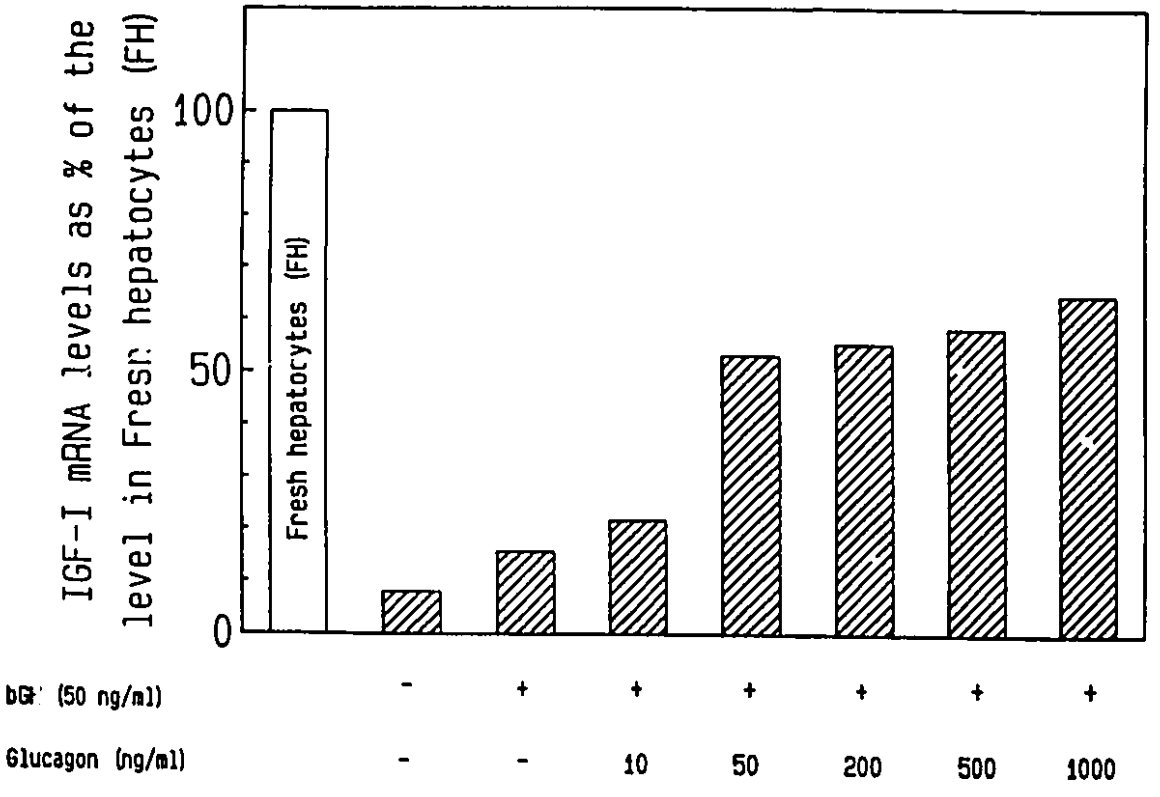

kb
28 S
18 S
7.0
1.8
1.1
0.7
FH
bGH (50 ng/ml) - + + + + + +
Glucagon (ng/ml) - - 10 50 200 500 1000

Figure 3

3 B. Quantification of Northern blot (A) by densitometric scanning of 0.7-1.1 kb and 7.0 kb transcripts. Levels of IGF-I mRNA (sum of the levels of their major transcripts) in different samples are expressed as percentage of IGF-I mRNA level in freshly isolated hepatocytes which is normalized to 100%.

Figure 3 B


IGF-I mRNA levels as % of the level in Fresh hepatocytes (FH)
100
50
0
Fresh hepatocytes (FH)
bGH (50 ng/ml)
- + + + + + +
Glucagon (ng/ml)
- - 10 50 200 500 1000

level of IGF-I mRNA induced by combining glucagon and bGH was observed to be 60% of that in freshly isolated hepatocytes, it is possible that there are other factors required for achieving and maintaining the IGF-I mRNA levels observed in freshly isolated hepatocytes.

Effect of bGH and/or glucagon on IGF-BPs secreted by hepatocytes

Hepatocytes have been shown to secrete IGF-BPs into their culture medium (36-38). We therefore examined the influence of bGH and glucagon on IGF-BPs secreted into the culture medium after 24 h incubation. As noted in Fig. 4, IGF-BPs, assessed by charcoal binding assay, were secreted by hepatocytes cultured in DMEM/F12 medium containing nutritional factors only. Glucagon augmented IGF-BP secretion, whereas bGH stikingly inhibited their secretion. In the presence of glucagon the inhibitory effect of bGH was markedly attenuated. This is confirmed for three separate experiments as summarized in Table 3.

Western ligand blotting

In order to determine the different species of IGF-BPs secreted by hepatocytes and the effect of bGH and/or glucagon on these BPs, conditioned media were subjected to Western ligand blotting as described in Materials and Methods. The major species of IGF-BPs secreted were 24K and 30-34K, the latter being thought to constitute several species (39). Again

Figure 4. Effect of bGH and/or glucagon on IGF-BPs secreted by cultured rat hepatocytes after 24 h of incubation

Different volumes of conditioned media from hepatocytes treated without or with bGH and/or glucagon were assayed for [^{125}I]IGF-I binding activity by the charcoal binding assay described in Materials and Methods. This experiment is representative of three experiments, all of which showed comparable results.

Figure 4

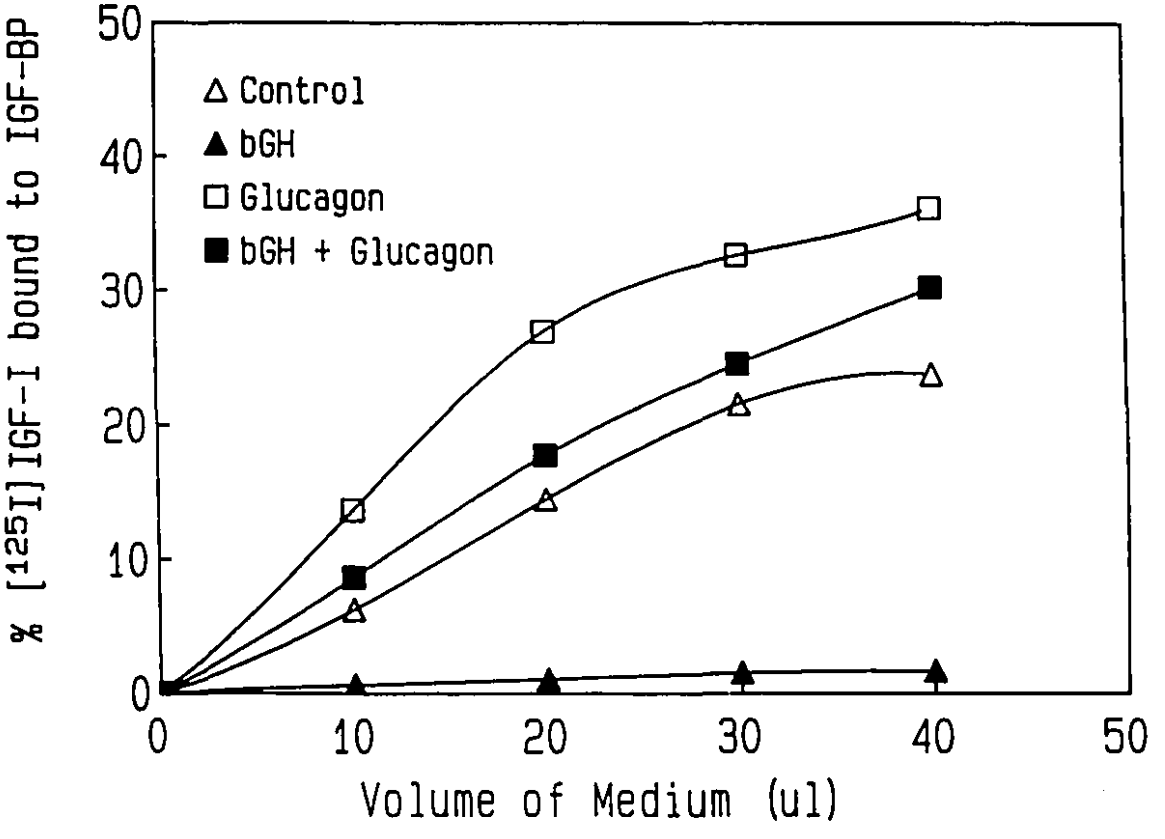

% [125I]IGF-I bound to IGF-BP
50
40
30
20
10
0
0
10
20
30
40
50
Volume of Medium (ul)
Control
bGH
Glucagon
bGH + Glucagon

Table 3. Effect of bGH and/or glucagon on IGF-BP accumulation in media from cultured rat hepatocytes

Treatment	IGF-BP (% [^{125}I]IGF-I bound)
Control	16.0 ± 3.0
bGH	4.4 ± 4.8
Glucagon	29.9 ± 3.0[a]
bGH + glucagon	23.1 ± 4.8[b]

Hepatocytes were prepared, plated, and incubated before treatment as described in Materials and Methods. After 24 h of treatment with either no additions (control) or bGH and/or glucagon the incubation media were collected and assayed for IGF-BP content using 20 ul and 40 ul aliquots of medium. Each value is the mean ± SD of three separate incubations.

[a] $P < 0.03$ compared to control.
[b] $P = 0.15$ compared to control.

glucagon appeared to stimulate IGF-BP secretion, whereas bGH markedly inhibited their secretion. In the presence of glucagon the inhibitory effect of bGH on all species was reversed (Fig 5).

Time course of bGH and/or glucagon effect on IGF-BP(s) secretion by hepatocytes

The secretion of IGF-BPs into the medium increased with time in control and glucagon-treated cells (Fig 6). In the presence of bGH the secretion of IGF-BPs was inhibited. This was not evident at 6 h of incubation with bGH but became noticeable at 12 h. The inhibitory impact was markedly evident by 24 h of incubation. The apparent decrease in binding proteins from 12 to 24 h raises the possibility that IGF-BPs are metabolized by hepatocytes over this time interval.

Figure 5. Effects of bGH and/or glucagon on different species of IGF-BPs secreted by cultured hepatocytes after 24 h of incubation

Conditioned media from hepatocytes treated without or with bGH and/or glucagon for 24 h were concentrated 10 fold using Centricon-10 microconcentrators. After electrophoresis and transfer of the concentrated media samples, Western ligand blotting was carried out using [^{125}I]IGF-I as described in Materials amd Methods. Lanes 1 and 2 contained, respectively, 50 ug and 100 ug media proteins. Autoradiographic exposure was for 4 days at -70 C. This experiment is one of three different experiments, all of which gave comparable results.

Figure 5

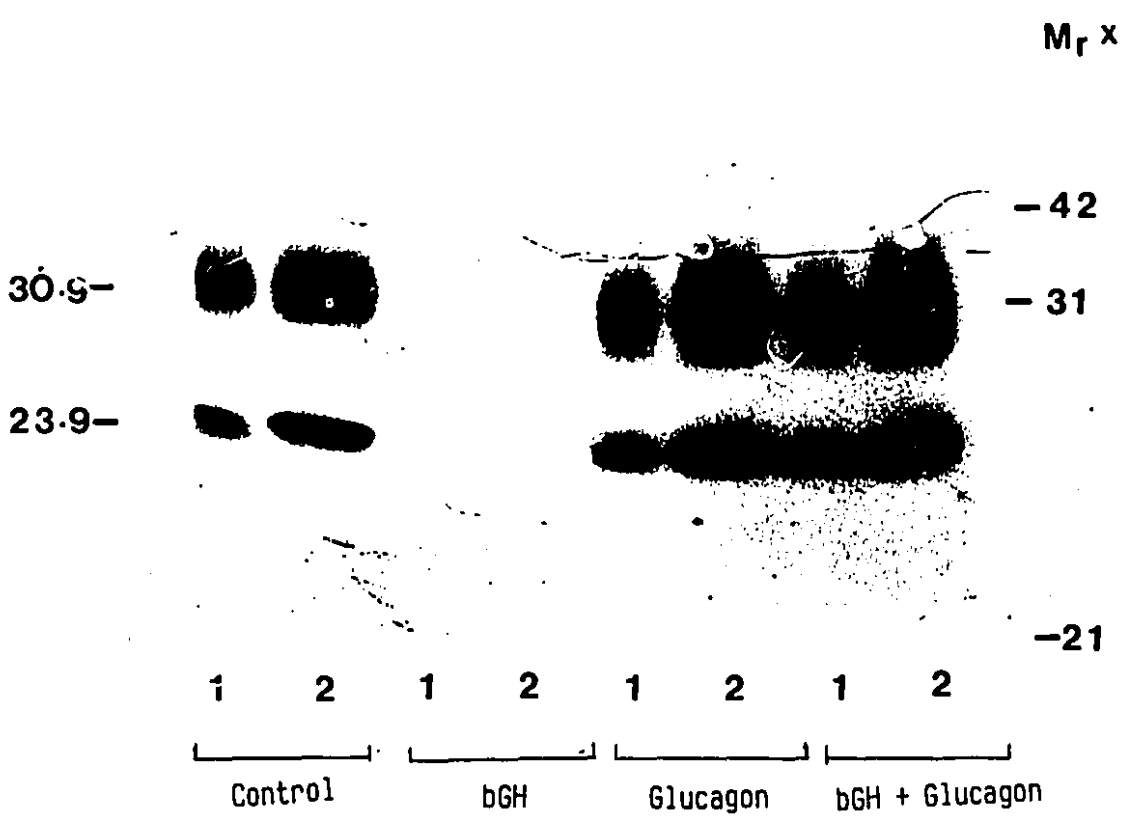
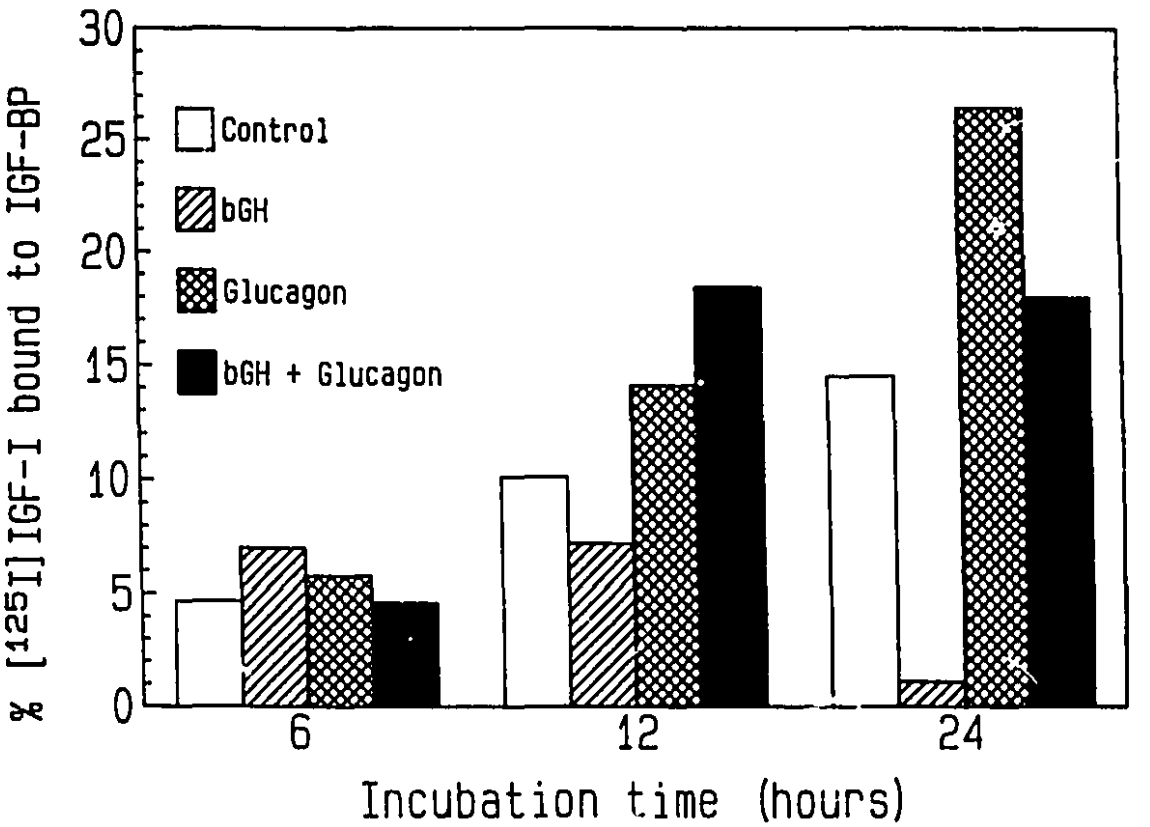

$M_r \times 10^{-3}$
30·9–
23·9–
–42
– 31
–21
1 2 1 2 1 2 1 2
Control
bGH
Glucagon
bGH + Glucagon

Figure 6. Time dependent effect of bGH and/or glucagon on IGF-BPs secreted by cultured rat hepatocytes

[^{125}I]IGF-I binding activity was determined by the charcoal binding assay described in Materials and Methods using 20 ul conditioned media. Hepatocytes were treated without or with bGH and/or glucagon for various times as indicated in the figure.

Figure 6.


30
25
20
15
10
5
0
% [125I] IGF-I bound to IGF-BP
Control
bGH
Glucagon
bGH + Glucagon
6
12
24
Incubation time (hours)

DISCUSSION

Several in in vivo studies have demonstrated that IGF-I mRNA decreases in hypophysectomy and is returned towards normal by GH treatment (4,5,8). In these studies multiple species of mRNA were observed, using Northern blotting techniques, corresponding principally to three transcripts of 7.0 kb, 1.8 kb, and 0.7-1.1 kb. Regulation of IGF-I mRNA levels by GH has also been seen in nonhepatic tissues (4), but this appears to be less marked an effect than that seen in liver (4,8).

Previous in vitro studies on cultured rat hepatocytes have demonstrated that GH treatment stimulated the accumulation of IGF-I mRNA (6,40) and IGF-I secretion into the culture medium (6,36-38). Although Johnson et al (6) reported a biphasic response of IGF-I mRNA accumulation to bGH, our studies suggest maximum stimulation by 12 h and a decline by 24 h of incubation.

Glucagon alone modestly stimulated IGF-I mRNA transcript accumulation to an extent comparable to that previously reported for insulin alone (6). Of particular interest is our observation that glucagon markedly potentiated the stimulatory effect of bGH. The effect of glucagon to stimulate IGF-I gene expression and IGF-I production, and to augment markedly that observed with bGH alone, has not been previously reported. Johnson et al (6) reported 3-fold stimulation of IGF-I mRNA in hepatocytes by GH. No further stimulation was observed when GH and insulin were added together. In this study we note a 10-fold stimulation of IGF-I mRNA by GH in the presence of

glucagon. Whereas the combined effect of glucagon and bGH on IGF-I mRNA levels significantly exceeded the sum of their individual effects, this was not so for IGF-I secretion. The disproportionate augmentation of IGF-I mRNA levels vs. amounts of IGF-I secreted may reflect the generation of some mRNA species that are poorly translated. Other possibilities cannot be ruled out including the coproduction of factor(s) inhibitory of IGF-I mRNA translation or stimulatory of IGF-I metabolism.

The mechanism of glucagon's effect requires delineation, since glucagon has been shown to stimulate inositol phosphate production as well as that of cAMP in hepatocytes (41). cAMP has been implicated in the stimulation of IGF-I production as evidenced in studies on porcine granulosa cells (42). Further studies are also needed to distinguish between the effect on IGF-I mRNA accumulation via the alteration of transcription rate vs. the prolongation of mRNA half-life.

Trophic hormones have been shown to stimulate IGF-I production in various cell types (45-48). In all instances the stimulation of cAMP production occurs concomitantly. This appears to be true for FSH and LH acting on porcine granulosa cells (42) and ACTH acting on cultured bovine adrenal cells (43). TSH also stimulated the concomitant production of IGF-I and cAMP. In these latter studies a role for cAMP as a mediator of augmented IGF-I production was supported by the demonstration that agents which induced increased intracellular levels of cAMP in thyroid cells also increased IGF-I secretion

(44). It is possible that many trophic hormones activate both cAMP production and another pathway (i.e. a sequence involving protooncogenes) leading to IGF-I production and growth. This seems to explain the effect of EGF on growth-arrested rat H4IIE hepatoma cells (45). In the case of liver the stimulus may not cohere in a single hormone but in two different agents, namely, glucagon and GH. In this regard it is of interest to note that glucagon has been implicated as a trophic factor for regenerating liver (46).

The rat liver has been shown to secrete IGF-BPs (2,36-39). By Western ligand blotting the major species found were 24K and 30-34K, the latter thought to constitute several species (39). Our data are substantially the same as that of Hossenlopp et al (39). A low mol wt IGF-BP (24K) in our samples may be similar to that reported in human fibroblasts (32). Earlier observations suggested that GH had little or no effect on hepatic binding protein production (2). Our study has demonstrated a dramatic time-dependent inhibitory influence of GH on IGF-BP secretion into the culture medium. This effect was seen for all the BP species identified and is consistent with our earlier observations that microsomes of liver from hypophysectomized rats had considerably more IGF-BPs associated with them than microsomes from normal animals (34).

The effect of glucagon alone was only modestly evident, presumably because control cells secreted significant levels of IGF-BPs. However, when added along with bGH, glucagon substantially reversed the inhibitory effect of bGH on BP

secretion. Again, all species of IGF-BPs secreted appeared to be comparably affected. Our hepatocyte cultures might be viewed as reflecting the in vivo hypophysectomized state, where we have previously noted that liver-associated BP levels were augmented (34). This may reflect the depression of BP synthesis as suggested by the observation of Margot et al (20), wherein mRNA levels for a rat fetal BP were found to be greatly augmented after hypophysectomy. The inverse relationship between GH status and the level of IGF-BP-1 (47) and IGF-BP-2 (48) has recently been clearly documented. Also Lewitt and Baxter (49) showed that the secretion of an IGF-BP by cultured human fetal liver explants was stimulated about 90% by glucagon. Thus the stimulatory effect of glucagon, which we have observed, occurs in higher species as well and may be general phenomenon of significance.

Contrary to glucagon's effect on hepatocytes, FSH has been shown to inhibit IGF-BP secretion by granulosa cells (50) possibly via cAMP production. This suggests that cAMP may have different effects on the production or that IGF-BPs secreted by hepatocytes are different from IGF-BPs secreted by granulosa cells. It is not possible to deduce as yet the type of IGF-BPs present in our samples, as Western ligand blotting does not allow us to reliably distinguish between IGF-BP-1 and IGF-BP-2 (51).

Since IGF-BPs, in the size range secreted by hepatocytes, have been implicated in potentiating IGF-I binding and action

(52), it is possible that glucagon's hepatotrophic effect may be mediated by its augmentation of BP levels. This is rendered more plausible perhaps by the consideration that rat liver contains no type I IGF receptors (53). Thus, IGF-I action on rat liver may follow the binding of IGF-I to BP and the incorporation of this complex into hepatocytes. With recent progress in purifying and characterizing IGF-BPs (9) this hypothesis may be within reach of rigorous testing.

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CHAPTER 3

OCTREOTIDE INHIBITS INSULIN-LIKE GROWTH FACTOR-I HEPATIC GENE EXPRESSION IN THE HYPOPHYSECTOMIZED RAT: EVIDENCE FOR A DIRECT AND INDIRECT MECHANISM OF ACTION

PREFACE TO CHAPTER 3

The potentiating effect of glucagon on GH stimulation of IGF-I production and IGF-I gene expression was observed in vitro in hepatocytes. This chapter discusses a similar effect of glucagon which is observed in vivo in hypophysectomized rat.

The long acting somatostatin analog octreotide (SMS) has a direct inhibitory effect on human growth hormone (hGH) release by cultured pituitary tumor cells from acromegalic patients. The serum IGF-I levels were reduced by octreotide in acromegalic patients. This study was undertaken to investigate if octreotide had any direct effect on stimulation of IGF-I production by GH in hypophysectomized rats.

Octreotide was found to inhibit GH effect on cirulating levels of IGF-I and hepatic IGF-I mRNA levels in hypophysectomized rats. Serum glucagon levels were found to be lower in hGH plus octreotide administered hypophysectomized rats compared to hGH treated hypophysectomized rats. Octreotide was also found to have a direct inhibitory effect on bGH induced IGF-I mRNA stimulation in cultured rat hepatocytes. These data indicate that glucagon also appears to be an important modulator of GH's stimulatory effect on circulating levels of IGF-I production and hepatic IGF-I gene expression in vivo. Octreotide inhibits GH's effect on the stimulation of circulating levels of IGF-I and hepatice IGF-I gene expression partly by lowering glucagon level in blood and partly by a direct effect on IGF-I mRNA levels.

ABSTRACT

Somatostatin and somatostatin analogues are known to interact with the growth hormone (GH)-insulin-like growth factor (IGF)-I axis by inhibiting GH secretion and consequently hepatic IGF-I production. Indirect evidence suggests that octreotide, a somatostatin analogue, reduces serum IGF-I levels relatively more than expected from GH reduction, implying a GH-independent pathway of action. To study the role of octreotide in the regulation of IGF-I production, independently of endogenous GH, we used the hypophysectomized (hypox) rat to measure hepatic IGF-I expression and also employed cultured rat hepatocytes to examine whether octreotide has any direct effect on the production of IGF-I. Forty male hypox Sprague-Dawley rats were randomized into 4 groups to receive daily injections for 3 days of either saline, hGH (100 g), octreotide (100 g twice), or both hGH (100 g) and octreotide (100 g twice). GH stimulated serum IGF-I levels to 104 ± 10 ug/liter as compared to saline (26 ± 2 g/l). Octreotide alone had no effect but combining octreotide and hGH significantly reduced the hGH-induced rise in the IGF-I levels (52 ± 6 g/l). The relative expression of hepatic IGF-I in the rats treated with hGH increased by a 4-fold compared to that in the saline-treated rats. Octreotide administered simultaneously with hGH potently blocked the hGH-induced IGF-I expression to control levels.

In cultured hepatocytes, IGF-I mRNA levels maximally stimulated by combining bGH and glucagon were significantly

inhibited in the presence of octreotide at low concentrations (0.3 and 3 ng/ml) by 25% and 45%, respectively. In contrast, high concentrations of octreotide (30 and 300 ng/ml) had no significant effect on IGF-I mRNA abundance.

We conclude that 1) octreotide inhibits IGF-I serum levels and hepatic gene expression in the hypox rats; 2) octreotide can inhibit partially the direct effects of GH and glucagon on hepatic IGF-I production.

INTRODUCTION

Since the development of the somatomedin hypothesis, Insulin-like growth factor (IGF)-I is considered as an important mediator of growth hormone (GH) action, particularly during the postnatal period (1,2). It is now well established that circulating GH of pituitary origin interacts with a specific hepatic receptor stimulating the synthesis and secretion of IGF-I. The effect of GH on hepatic IGF-I synthesis has been shown to be due to changes in IGF-I mRNA. Thus, hypophysectomy of rats is associated with decreased hepatic IGF-I mRNA, and GH replacement therapy either partially or completely restores IGF-I mRNA levels (3-7). The stimulatory effect of GH on hepatic IGF-I mRNA levels has been shown to be a direct effect on hepatocytes (8,9). Somatostatin and somatostatin analogues interact with the pituitary-somatomedin axis by inhibiting GH secretion and consequently reducing IGF-I production (10,11). However, it has been suggested that in several acromegalic patients treated with the long-acting somatostatin analogue, octreotide, serum IGF-I levels were reduced relatively more than circulating GH concentrations (12-15). Moreover, we recently reported that treatment with octreotide decreases serum IGF-I levels in type 1 diabetic patients without altering significantly plasma GH concentrations (16). These findings suggested that octreotide treatment leads to a reduction in circulating IGF-I levels by

at least more than inhibition of pituitary GH production. We have therefore used the hypophysectomized (hypox) rat to investigate the effects of octreotide independently of endogenous GH and we have also employed hepatocytes maintained in a serum free medium to examine whether octreotide has any direct effect on the production of IGF-I. Our results indicate a previously unrecognized action of octreotide in inhibiting the expression of hepatic IGF-I mRNA and the secretion of IGF-I. Furthermore, this inhibitory action of octreotide appears to be mediated in part by a direct effect at the hepatocyte level.

Methods

Male Sprague Dawley rats, hypophysectomized at 90-100 g BW, were obtained from Charles River Canada (St. Constance, Quebec, Canada) and acclimatized in individual cages for approximately 10 days. Rats were fed ad libitum. To confirm that hypophysectomy was successful, the animals were weighed daily. Only rats that demonstrated less than 3g weight gain per week and had no evidence of pituitary remnants at the time of sacrifice were considered hypox. The rats were randomized into 4 groups with 10 animals in each group. One group received two daily (at 0800 h and 1600 h) subcutaneous injections of 0.9% saline. The second group received two daily subcutaneous injections of octreotide (100 g/100 g BW) (generously provided by Karen Gallant, Sandoz Canada, Dorval, Canada). The third group received a subcutaneous injection at 0800 h of 100 g/100 g BW human (h) GH (a Kind gift from Michael Cronin, Genentech USA, South San Francisco, California, USA) and a saline injection at 1600 h. In the fourth group, both octreotide and hGH (100 g/100 g BW each), were administered simultaneously at 0800 h, and octreotide alone was injected at 1600 h. The doses of hGH and octreotide used in the present experiments were based on results of previous studies demonstrating consistent stimulation and inhibition respectively of serum IGF-I (17,18). Treatments were continued for 3 days. All animals were sacrificed on the third day at

1300 h. Trunk blood was centrifuged immediately after coagulation and serum was frozen at - 20 C for later analysis. The liver tissue was rapidly removed, immediately frozen on dry ice and stored at - 70 C.

IGF-I radioimmunoassay (RIA)

Human recombinant IGF-I (Amgen Co, Thousand Oaks, California) was used for iodination and standards, while human IGF-I antibody (raised by L.E. Underwood and J.J. Van Wyk, University of North-Carolina, Chapel Hill, NC) was donated by the U.S. National Hormone and Pituitary Program. IGF-I was measured in rat serum after extraction in glycine-hydrochloric acid as previously described (16). Intra-assay and interassay coefficients of variation were 4.2% and 10.4% respectively.

Insulin and glucagon RIAs

Serum immunoreactive insulin (IRI) and plasma glucagon were measured by conventional double antibody RIA methods. IRI was measured with a kit from Immunocorp (Montreal,Canada) using a rat insulin standard from Novo (Mississauga, Canada). Glucagon was determined with a kit from Diagnostic Products Corporation (Los Angeles,CA) using an antibovine glucagon rabbit serum. The intra and interassay coefficients of variation were less than 9% in both assays.

Hormones and chemicals

Bovine GH (USDA-bGH-B-1, 1.4 IU/mg) was kindly supplied by the USDA Animal Hormone Program (Beltsville, MD). IGF-I antiserum (UBK 487) was kindly provided through the Hormone Distribution Program of the NIDDK (Bethesda, MD), courtesy of DRS. J.J. Van Wyk and L. Underwood. IGF-I was a generous gift from Ciba-Geigy Ltd. (Basel, Switzerland) and Chiron Corp. (Emeryville, CA). Ornithine, L-lactic acid, glucagon, selenium, ethanolamine, EGTA, protamine sulfate, Denhardt's solution (50x) and salmon sperm DNA were purchased from Sigma Chemical (St-Louis, MO). Collagenase was from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12 Nutrient mixture, and fetal calf serum (FCS) were from GIBCO (Grand Island, NY). Tissue culture plates (100 mm), penicillin, streptomycin, and Fungizone were from Flow Laboratories (Rockville, MD). Collagen (Vitrogen 100) was from Collagen Corporation (Palo Alto, CA). Carrier free ^{125}I was purchased from New England Nuclear Corp. (Boston, MA). Formamide, guanidinium isothiocyanate, and Cesium chloride were purchased from IBI (New Haven, CT). Random primers DNA labeling system, ECoR I restriction enzyme, and RNA molecular size markers were from BRL (Gaitherburg, MD). [^{32}P]dCTP (3000 Ci/mmol) and Hybond-N nylon membrane were purchased from Amersham Co. (Arlington Heights, IL).

Preparation of primary hepatocyte cultures

Hepatocytes were isolated from male Sprague Dawley rats

weighing 180-200 g by perfusion in in situ with collagenase as described previously (19,20). At the end of perfusion the liver was removed and placed in DMEM/F12 medium containing 10 mM HEPES and 20 mM $NaHCO_3$ supplemented with 10% fetal bovine serum, 500 IU/ml penicillin, 500 g/ml streptomycin, and 1.25 g/ml Fungizone. An aliquot of cell suspension was diluted 5:1 with Trypan blue to determine cell viability, which was 80% at the onset of culture. Cells were plated at a density of 5×10^6 cells/100 mm culture plates. The plates were previously coated with a 1% solution of collagen and incubated at 37 C in a humidified atmosphere (5% CO_2)). After overnight attachment, the culture medium was changed to a serum-free medium supplemented with 0.4 mM ornithine, 2.25 g/ml L-lactic acid, 2.5×10^{-8} M selenium, and 1×10^{-8} M ethanolamine (20). Thereafter the media were changed daily throughout the duration of the experiment.

Preparation of RNA

Total RNA from rat liver samples and from cultured rat hepatocytes was prepared by the guanidinium isothiocyanate-Cesium chloride technique (21). Liver samples and hepatocytes were homogenized in 5 vol of 4 M guanidinium isothiocyanate buffer containing 0.1 M Tris-HCl (pH 7.5) and 1% mercaptoethanol. Hepatocyte monolayers were lysed in the above buffer (2 ml/100 mm culture plate). The homogenates or cell lysates were layered on 4 ml 5.7 M cesium chloride

containing 0.01 M EDTA (pH 7.5) in 13 ml Beckman Ultra Clear centrifuge tubes. The samples were centrifuged at 33,000 rpm in a Beckman Ultracentrifuge (model LM8) using an SW40 rotor for 24 hours. The RNA pellet was dissolved in 0.3 M sodium acetate buffer pH 5.2 and precipitated with 2.2 vol ethanol at - 20 C overnight. The pellet was dried in a speedvac and stored at - 70 C.

Northern blot analysis

Total RNA samples (40 g/ml) were denatured in 50% formamide and 2.2 M formaldehyde at 65 C for 15 min. The samples were electrophoresed through 1.5% agarose, 2.2 M formaldehyde denaturing gel using 20 mM 3-(N-morpholino) propanesulfonic acid buffer containing 8 mM sodium acetate and 1 mM EDTA (pH 8.0) (22). Ribosomal RNAs (28S and 18S) were visualized under UV light to ensure the integrity of RNA samples. RNA was transfered to Hybond-N membranes by capillary tranfer using 1 M sodium chloride. RNA was fixed to the membrane by baking at 80 C for 2 h. One lane containing RNA molecular size markers was removed from the rest of the membrane and stained with 0.04% methylene blue (22).

IGF-I cDNA probe

A rat IGF-I cDNA consisting of a 422 basepair fragment, coding for the pre-, B, C, A, D, and part of the E domains of IGF-I peptide, inserted in plasmid PUC 19, and tranfected in Escherichia coli was kindly provided by Drs. C. Roberts and D. LeRoith of the NIH. The IGF-I cDNA insert was isolated by ECoR

I restriction enzyme digestion, followed by electrophoresis in low melting point agarose. IGF-I cDNA (20 ng) was labeled with [^{32}P]dCTP by a random primer labeling technique (23) using the procedure described in the BRL random primer labeling system. The probe was labeled to a specific activity of 10^9 dpm/ g DNA.

Hybridization

Membranes were prehybridized in 5x SSPE (1x SSPE = 180 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, pH 7.4), 50% formamide, 5x Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 100 g/ml salmon sperm DNA for 2 h at 42 C. The prehybridization solution was replaced by a fresh hybridization solution containing the labeled probe at a final concentration of 1 x 10^6 cpm/ml, and the incubation was continued for 16-18 h at 42 C. The blots were washed twice at room temperature in 2x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 0.1% SDS for 30 min each and once at 60 C in 1 x SSC, 0.1% SDS for 15 min. The blots were exposed to Kodak X-AR film (Eastman Kodak Co., Rochester, NY) at -70 C for 2-3 days.

Densitometry quantitation

Each RNA sample was quantitated in triplicate by dot blot analyses. The intensity of the signal of each sample was obtained using an LKB Ultrascan XL enhanced laser densitometer.

Statistical analysis

Differences between groups were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range test or unpaired Student's t-test. Results are given as mean values ± SE.

RESULTS

Body weight, Liver weight and Kidney weight

The effects of octreotide, hGH or both on body weight and liver and kidney weights for the 3-day treatment period in hypox rats are shown in Table I. The different treatments had no significant effect on either BW, liver or kidney weights.

Serum IGF-I

Hypox rats treated with saline had low serum IGF-I levels (26 ± 2 g/l). Treatment of rats with hGH significantly increased serum IGF-I concentrations (104 ± 10 g/l). Administration of octreotide in comparison with saline had no effect on serum IGF-I levels (23 ± 2 g/l). However, administration of octreotide simultaneously with hGH significantly reduced to about 50% the hGH-induced rise in IGF-I levels (52 ± 6 g/l)($P < 0.01$, Fig 1).

Serum IRI and plasma glucagon

The effects of octreotide, hGH or both on serum IRI and plasma glucagon are shown in Table II. The treatments had no effect on IRI levels. However, plasma glucagon levels appeared to be lowered by administration of hGH combined with octreotide as compared to the group of animals receiving hGH alone (64 ± 9 vs 94 ± 10 ng/l respectively, $P = 0.012$).

Table I. Effects of octreotide, hGH, or both on body, liver and kidney weights in hypophysectomized rats

Treatment	Body weight (g)	Liver weight (g)	Kidney weight (g)
Saline	98 ± 2	4.00 ± 0.2	0.78 ± 0.02
Octreotide	100 ± 3	3.60 ± 0.1	0.76 ± 0.02
hGH	99 ± 2	3.93 ± 0.2	0.78 ± 0.03
Octreotide + hGH	98 ± 2	3.68 ± 0.2	0.82 ± 0.04

Fig.1. The effect of administration of octreotide, hGH, or both on serum IGF-I concentrations in the hypox rat.

Rats received daily injections for 3 days, as described in Methods. Values are expressed as means ± SE. ($P < 0.01$, GH + octreotide vs GH and GH vs saline).

Figure 1

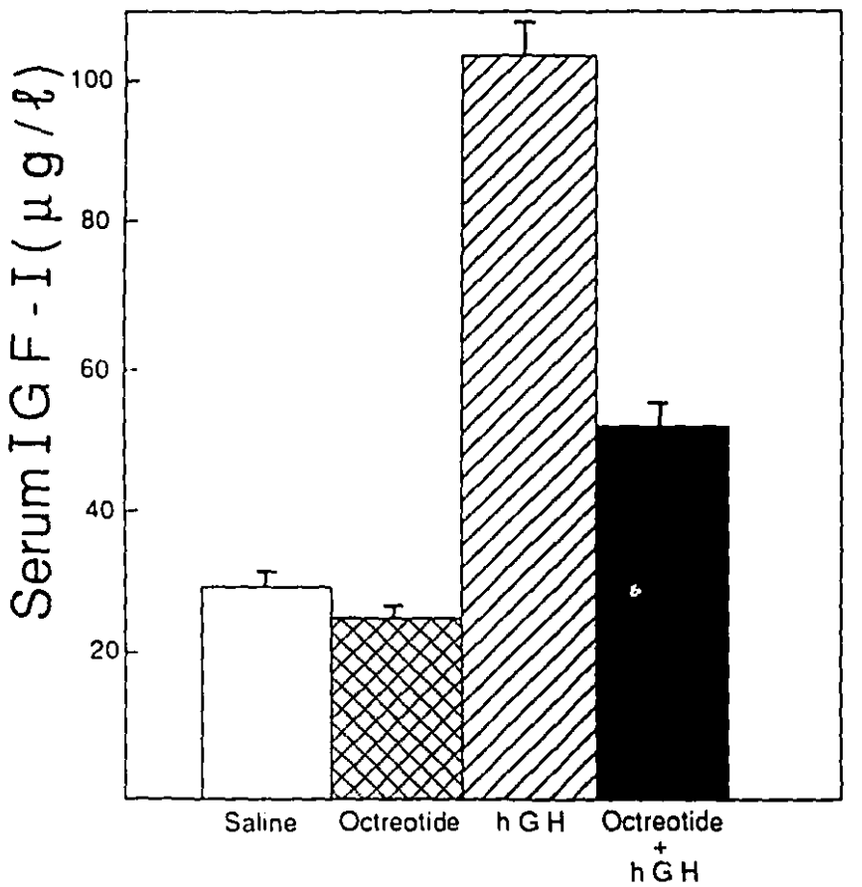
Serum IGF-I (μg/ℓ)
100
80
60
40
20
Saline
Octreotide
h G H
Octreotide + h G H

Table II. Effects of Octreotide, hGH or both on serum insulin (IRI) and plasma glucagon levels in hypophysectomized rats

Treatment	IRI (pmol/l)	Glucagon (ng/l)
Saline	170 ± 40	77 ± 13
Octreotide	125 ± 20	60 ± 25
hGH	148 ± 32	94 ± 10[a]
Octreotide + hGH	119 ± 17	64 ± 9[a]

[a] $p = 0.012$

Hepatic IGF-I mRNA expression

Administration of hGH alone to hypox rats significantly increased IGF-I mRNA abundance in the liver (Fig.2). The relative expression of IGF-I in the rats treated with hGH increased by 4-fold compared to that in the saline-treated rats ($P < 0.01$, Fig.3). Octreotide alone had no effect on IGF-I mRNA levels, but when administered simultaneously with hGH it inhibited significantly ($P < 0.01$) IGF-I mRNA increase (Figs 2 and 3). The relative expression of IGF-I in rats treated with both hGH and octreotide was similar to that in the control saline-treated rats (Fig.3).

Effects of octreotide on IGF-I expression in cultured hepatocytes

We examined in cultured hepatocytes the influence of octreotide on IGF-I mRNA levels maximally stimulated by combining glucagon and bGH. The data in Table III indicate that in the presence of bGH and glucagon, octreotide at low concentrations (0.3 and 3 ng/ml), significantly inhibited IGF-I mRNA (by 25% and 55%, respectively). In contrast, high concentrations of octreotide (30 and 300 ng/ml) had no significant effect on IGF-I mRNA abundance (Table III).

Fig.2. Effects of administration of octreotide, hGH, or both on liver IGF-I mRNA levels in hypox rats.

2 A. Autoradiogram of Northern blot of total RNA from hypox rat liver (Lanes 1-4) and normal control rat liver (lane 5). Forty micrograms of total RNA was size fractionated on 1.5% agarose, 2.2 M formaldehyde gel, transfered to Hybond-N membrane and hybridized with [^{32}P] labeled IGF-I cDNA probe. The membrane was exposed to Kodak X-AR film for 3 days at -70 C.

2 B. To ensure that equal amounts of total RNA was applied to each lane, IGF-I probe was stripped from membrane A and the same membrane was rehybridized with ^{32}P-labeled GAPDH cDNA probe. The membrane was exposed to Kodak X-AR film for 24 h at -70 C.

Figure 2.

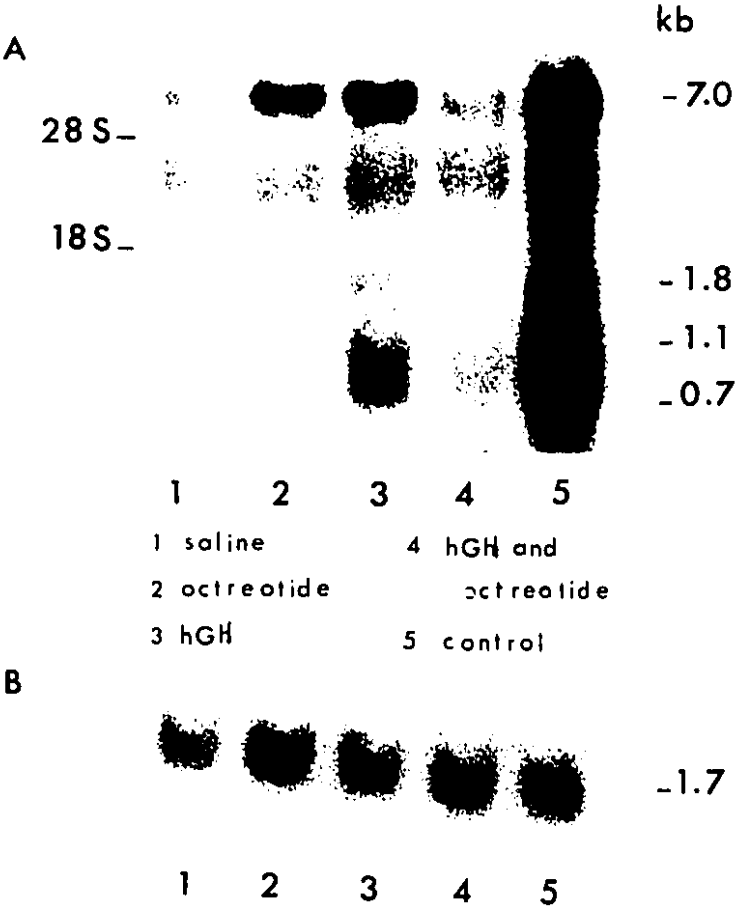
kb
A
28 S
18 S
7.0
1.8
1.1
0.7
1 2 3 4 5
1 saline
2 octreotide
3 hGH
4 hGH and octreotide
5 control
B
1.7
1 2 3 4 5

Fig. 3. The effect of administration of octreotide, hGH, or both on hepatic IGF-I gene expression in the hypox rat.

Rats received daily injections for 3 days, as described in Methods. The relative expression of IGF-I, calculated from densitometry of dot blot data, has been expressed relative to that in normal rat liver (IGF-I mRNA level in normal rat liver is normalized to 100%). Values expressed are means ± SE of data obtained in three separate experiments. ($p < 0.01$, GH vs all other groups).

FIGURE 3

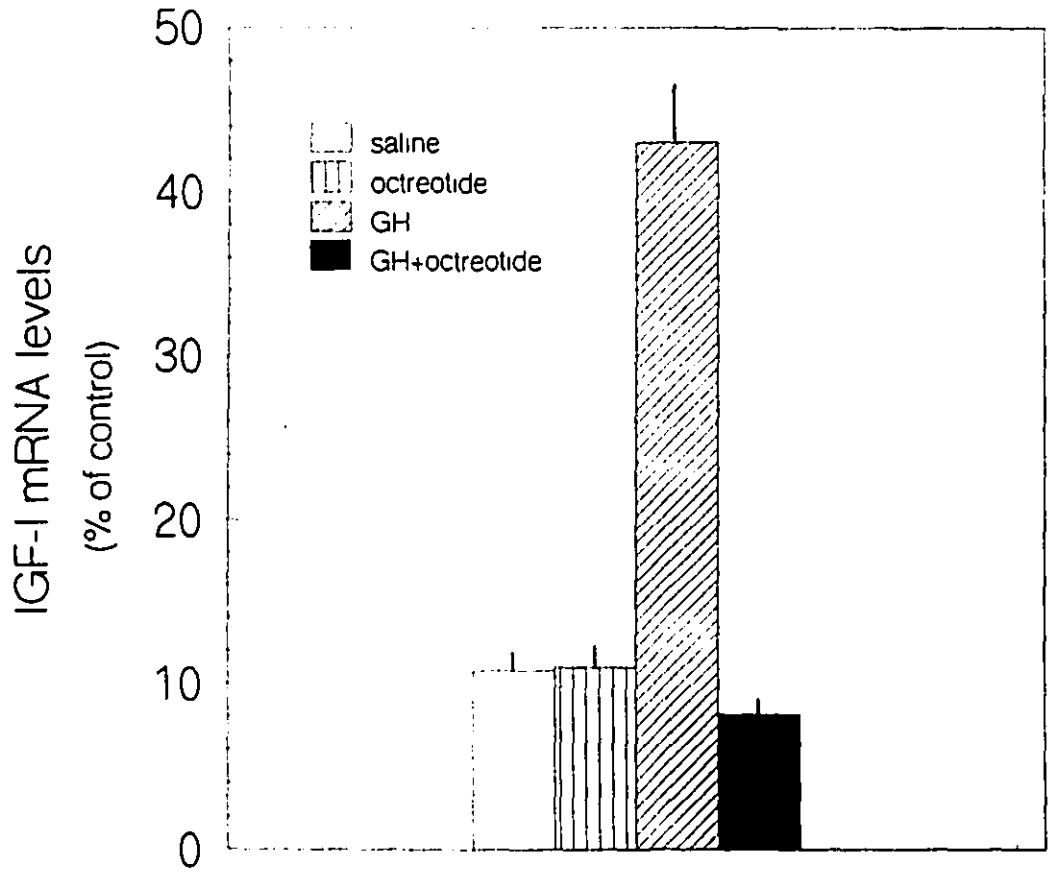
IGF-I mRNA levels
(% of control)
50
40
30
20
10
0
saline
octreotide
GH
GH+octreotide

Table III. Effects of Octreotide on IGF-I mRNA levels in cultured rat hepatocytes in the presence of bGH and glucagon

Concentration of octreotide (ng/ml)	IGF-I mRNA levels % of control
0	100
0.1	83.1 ± 18.7
0.3	75.1 ± 1.1
3.0	55.3 ± 5.9
30	99.9 ± 2.7
300	95.3 ± 2.0

Hepatocytes were treated with different concentrations of octreotide for 30 min before adding bGH (50 ng/ml) and glucagon (100 ng/ml). After 6 h of incubation with bGH and glucagon, total RNA was prepared from the cells, and dot blot analyses were performed as described in Materials and Methods. IGF-I mRNA was quantitated by densitometric scanning. Levels are expressed as percentage of control (mean ± SE).

DISCUSSION

Our studies have confirmed those of others (3-7, 24), indicating that GH administration to hypox rats leads to an increase in hepatic IGF-I mRNA and in circulating IGF-I levels. We have also shown that octreotide treatment inhibited the GH-induced rise in serum IGF-I, thus confirming the results of a recent study by Flyvbjerg et al (25). In this latter study, Flyvbjerg et al., using a treatment period of 11 days, reported that octreotide inhibited the GH-induced rise in serum IGF-I levels by 40% and reduced body growth of the hypox rats. In the present study, neither GH nor octreotide had an observable effect on whole body or organ growth presumably because of the short time period (3 days) of treatment. Taken together these observations suggest that octreotide had a primary inhibitory effect on hepatic IGF-I synthesis.

We therefore first examined in our experimental animals the effect of octreotide on hepatic IGF-I gene expression and then studied this expression in vitro using cultured rat hepatocytes. Of particular interest is our observation that octreotide markedly inhibited the in vivo stimulatory effect of GH on hepatic IGF-I gene expression in the hypox rat. This effect of octreotide has not been previously reported. We observed a 4-fold reduction of hepatic IGF-I mRNA abundance in rats treated with both octreotide and GH as compared to that in rats treated with GH alone.

The mechanisms of this antagonism of GH action by octreotide are unclear. The inhibition by octreotide of IGF-I synthesis may theoretically be mediated via reduction in insulin release. Circulating IGF-I levels may be decreased in insulinopenic diabetes. The decrease in hepatic IGF-I synthesis results from reduction in IGF-I mRNA levels (26-29). Insulin treatment of diabetic rats restores GH-stimulated IGF-I mRNA levels (28,29). In addition to its GH modulating effect, insulin has been reported to increase IGF-I mRNA in cultured hepatocytes, thus, suggesting a direct effect (8). In line with the study of Flyvbjerg et al (25), our results showed that serum insulin levels were not significantly modified by octreotide treatment. However, because of the high variability in insulin results, we cannot rule out the possibility that octreotide may have altered insulin secretion or dynamic responses and that this alteration was one factor in IGF-I inhibition. On the other hand, we found that plasma glucagon levels were significantly lowered by octreotide treatment. Interestingly, glucagon has been recently reported (30) to stimulate hepatic IGF-I gene expression and to augment markedly the stimulatory effect observed with GH. Thus a possible mechanism of octreotide action leading to the inhibition of IGF-I gene expression could be mediated through its inhibition of glucagon release.

To determine whether part of the effect of octreotide on hepatic IGF-I expression may be exerted directly at the hepatocyte level, we measured the levels of IGF-I mRNA in

cultured hepatocytes in the presence of increasing concentrations of octreotide. Our results demonstrate that maximal stimulation of IGF-I mRNA by the combined effect of glucagon and GH was significantly reduced by octreotide. Interestingly, we have found that octreotide produced a biphasic dose-response pattern on IGF-I synthesis. Thus, maximal inhibition of stimulated IGF-I expression occurred at a concentration of 3 ng/ml while a concentration of 300 ng/ml was not inhibitory. The reason for this diminished effect of octreotide at high concentrations is not known, although similar effects have been reported previously in the study of somatostatin effects on rat thyroid follicular cells (31). One possibility is that at high concentrations, octreotide may down-regulate or uncouple its receptors from their postreceptor effectors.

The ability of octreotide to inhibit GH-stimulated hepatic IGF-I gene expression more markedly in the hypox rat than in cultured hepatocytes suggests that its direct effect on liver does not account for its entire inhibitory action. Rather octreotide may effect its full inhibition by decreasing glucagon levels on one hand and by directly interfering with the combined effects of circulating GH and glucagon at the level of the hepatocyte on the other hand.

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CHAPTER 4

PROTEIN KINASE C AND PROTEIN KINASE A ARE NECESSARY BUT NOT SUFFICIENT TO MEDIATE BOVINE GROWTH HORMONE (bGH) AND GLUCAGON EFFECT ON IGF-I mRNA STIMULATION IN RAT HEPATOCYTES

PREFACE TO CHAPTER 4

This chapter discusses the mechanisms underlying bGH and glucagon's synergistic effect on IGF-I mRNA levels in cultured rat hepatocytes.

As previously shown, glucagon and bGH were found to produce a synergistic effect on hepatic IGF-I mRNA stimulation. It was of an interest to know what other hormones produced a similar effect on stimulation of IGF-I mRNA levels in hepatocytes. T_3, oPRL, insulin, EGF and dexamethasone when each added in combination with bGH or glucagon showed no synergistic effect on stimulation of IGF-I mRNA levels in hepatocytes, indicating that GH and glucagon are the major regulatory hormones of hepatic IGF-I production. Like glucagon, IBMX and $(Bu)_2$cAMP produced a synergistic effect on IGF-I mRNA stimulation when combined with bGH. Whereas no synergistic effect was observed on IGF-I mRNA stimulation when PMA was added in combination with bGH. These data indicate that glucagon acts via elevation of cAMP levels rather than via PKC pathway to produce a synergistic effect on IGF-I mRNA stimulation when added in combination with bGH. GH has been shown to produce some of its effects via elevation of PKC levels. The synergistic effect of bGH plus glucagon on IGF-I mRNA stimulation was markedly inhibited in PKC depleted cells and also in the presence of inhibitors of PKC. No synergistic effect on stimulation of IGF-I mRNA level was observed by stimulating PKA and PKC levels,

indicating that synthesis of one or more protein(s) or factor(s) was required besides elevation of PKC and PKA levels to produce a synegistic effect on IGF-I mRNA stimulation in hepatocytes.

ABSTRACT

In cultured rat hepatocytes, bovine growth hormone (bGH) and glucagon individually stimulated IGF-I mRNA levels 1.8 to 2.5 fold but when combined, synergized to stimulated IGF-I mRNA levels by 10 to 12 fold [Kachra et al (1991) Endocrinology 128:1723-1730]. This unique synergy on IGF-I mRNA stimulation was not observed when T_3 (10 nM), oPRL (200 ng/ml), dexamethasone (100 nM), EGF (20 ng/ml) or insulin (100 nM), each was added in combination with bGH (50 ng/ml) or glucagon (100 ng/ml). To probe the mechanism of the bGH plus glucagon effect, the response to 3-isobutyl-1-methyl xanthine (IBMX), dibutyryl cAMP [$(Bu)_2$cAMP] and 4ß-phorbol 12ß -myristate 13α-acetate (PMA) were evaluated in rat hepatocytes. Like glucagon, the addition of IBMX (100 uM) or $(Bu)_2$cAMP (150 uM) stimulated IGF-I mRNA levels 1.8 to 2.0 fold; but in the presence of bGH (50 ng/ml), augmented IGF-I mRNA levels 10 to 12 fold. Stimulation by $(Bu)_2$cAMP (5 to 150 uM) in the presence of bGH (50 ng/ml) was dose dependent. PMA (5 to 500 nM) stimulated IGF-I mRNA levels 1.2 to 1.4 fold but displayed no synerigism when added with bGH. The stimulatory effect of bGH plus glucagon on IGF-I mRNA levels was inhibited: (a) 80% after 24 h preincubation with 10 uM PMA; (b) 90% by 500 nM staurosporine; (c) 82% by 100 uM sphingosine; and (d) 85% by 1 mM H_7. No synergistic effect on IGF-I mRNA levels was seen when combining PMA (100 nM) with glucagon (100ng/ml) or $(Bu)_2$cAMP (5 to 500 uM).

In summary, the major hormonal regulators of hepatic IGF-I mRNA levels appear to be GH and glucagon. Our observations further indicate that hepatic IGF-I mRNA levels are regulated by pathways involving protein kinase C and protein kinase A but must involve one or more additional factor(s).

INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a basic peptide of 70 amino acids which appears to mediate the growth promoting actions of growth hormone (GH) and plays an important role in postnatal and adolescent growth (1).The liver is the major organ responsible for the circulating levels of IGF-I (2). many other tissues (viz. kidney, heart, lung, testes, brain, muscles) also produce IGF-I (3) and express IGF-I mRNA (4-8). Since the level of IGF-I mRNAs in these tissues (5-7,9,10) as well as in cultured cells (11-20) has been shown to be GH dependent, it is likely that IGF-I functions in an autocrine/paracrine (3) as well as endocrine role.

In previous studies we showed that combition of bGH and glucagon synergistically augmented IGF-I mRNA levels by 10 to 12 fold in cultured rat hepatocytes (19). In vivo studies were also indicative of a role for glucagon in realizing the effect of GH on hepatic IGF-I mRNA levels (21). Glucagon was observed to stimulates the production of inositol phosphate as well as that of cAMP in rat hepatocytes (27). Furthermore PMA was found to mimick the effect of glucagon on hepatic steroid metabolism (23). Other studies point to a role for protein kinase C (PKC) in modulating hepatic IGF-I production. Thus GH has been shown to stimulate the formation of diacylglycerol (DAG) in canine kidney membranes (24), Ob1771 mouse preadipocytes (25) and rat hepatocytes (20, 26). This indicates that protein kinase C (PKC) could be a mediator of GH action. Thus the mechanism by

which glucagon synergizes with bGH may involve stimulation of PKC and/or protein Kinase A (PKA) acitivity. The present study was undertaken to investigate the relative contribution of the PKC and PKA pathways in the regulation of hepatic IGF-I mRNA levels by bGH and glucagon. The evidence obtained shows that activation of PKA and PKC are necessary but not sufficient and that one additional factor is required to achieve maximal augmentation of hepatic IGF-I mRNA levels by glucagon/bGH.

Materials and Methods

Hormones and Chemicals

Bovine growth hormone (bGH-B-1, 1.4 IU/mg) and Ovine prolactin (oPRL) were kindly supplied by USDA Animal Hormone Program (Beltsville, MD). 4ß-phorbol 12ß-myristate 13α-acetate (PMA), 4α-phorbol, 12, 13, didecanoate ($\alpha PDco_2$), 3-isobutyl-1-methyl xanthine (IBMX), N^6,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt, sphingosine, staurosporine, 1-(5-isoquinolinyl sulphonyl)-2-methyl piperazine (H7) free base, insulin, dexamethasone and 3,5,3'-triiodothyronine (T_3) and glucagon were purchased from Sigma Chemical Co. (St. Louis, Mo). EGF was obtained from Collaborative Research (Bedford, MA). The chemicals and reagents used for liver perfusion, hepatocyte cultures, RNA extraction and Northern blot analyses were obtained as indicated previously (19).

Hepatocyte cultures and hormonal stimulation

Hepatocytes were isolated from male Sprague Dawley rats by perfusion <u>in situ</u> with collagenase (27,28) and maintained in a serum free medium as described before (19). 72 h after plating, the hepatocytes were stimulated with various hormones and/or combinations for 6 h.

RNA extraction, Northern and dot blot analyses

At the end of each incubation period, total RNA was

extracted from the hepatocytes by the guanidinium isothiocyanate cesium chloride technique (29,30) as described in an earlier study (19). 40 ug of total RNA was size fractionated on a 1.5% agarose-2.2 M formaldehyde denaturing gel and transfered to a hybond-N-nylon membrane by capillary transfer as described previously (19). Dot blot analyses was carried out on 5 ug of total RNA in triplicate in a dot blot manifold (Bio-Rad) using the manufacturer's protocol. RNA was fixed to the membranes by heating in an oven at 80 C for 2 h. The membranes were first hybridized with a ^{32}P-labeled (2 x 10^6 cpm/ml) IGF-I cDNA probe (422 bp). To normalize IGF-I mRNA levels, the membranes were stripped and rehybridized with a ^{32}P-labeled (0.5 x 10^6 cpm/ml) GAPDH cDNA probe (750 bp). The membranes were stripped using the manufacturer's protocol for hybond-N-membrane. The cDNA probes were labeled to a specific activity of 10^9 dpm/ug DNA using random primer DNA labeling system. The hybridization and washing procedures were performed as described before (19).

Densitometric quantitation

IGF-I mRNA levels were quantitated by scaning the dot blots and all the transcripts in the Northern blots using an LKB Ultrascan XL enhanced laser densitometer. The results are expressed as mean ± S.D. of triplicate determinations of the ratio of IGF-I to GAPDH mRNA levels. The determinded levels of IGF-I mRNA in all the samples are expressed relative to the control value of 100%

Statistical analyses

Statistical analyses were carried out by paired t-test. Values < 0.05 was considered significant.

RESULTS

Effect of T_3, oPRL, dexamethasone and EGF on IGF-I mRNA levels

To investigate the role of various hormones on IGF-I mRNA levels, hepatocytes were treated with T_3, oPRL, dexamethasone and EGF in the presence or absence of bGH, glucagon or bGH plus glucagon. As shown in Table 1, T_3 and oPRL stimulated IGF-I mRNA levels 1.5 - fold and 1.4 - fold respectively in the presence of bGH. T_3 or oPRL had no effect on IGF-I mRNA levels in the presence or absence of glucagon or bGH plus glucagon. Dexamethasone showed 1.2 - fold stimulation of IGF-I mRNA levels in the presence or absence of glucagon but had no effect in the presence of bGH or bGH plus glucagon. None of the hormones tested showed any synergy when combined with bGH or glucagon on IGF-I mRNA levels.

Inhibition of IGF-I mRNA levels by insulin

Insulin was found to inhibit IGF-I mRNA levels 40 to 60% in the presence or absence glucagon or bGH plus glucagon as shown in Table 2. This was contrary to the stimulatory effect of insulin in hepatocytes reported by others (31,32) The culture medium in one study (31) contained T_3, dexamethasone and insulin, and in another (32) only dexamethasone and insulin. We thus investigated the effect of these and other additions on insulin's capacity to influence hepatocyte IGF-I mRNA (Table 2). In the presence of T_3, insulin minimally stimulated IGF-I

Table 1. Effect of T_3, oPRL, dexamethasone and EGF in the presence of bGH, glucagon and bGH plus glucagon on IGF-I mRNA levels in hepatocytes

Hormonal Treatment		IGF-I mRNA level % Control
Control		100
	+ T_3	107.2 ± 8.7
	+ oPRL	95.6 ± 6.3
	+ Dex.	129.3 ± 10.2
	+ EGF	52.5[c] ± 2.4
bGH		130.3 ± 7.0
	+ T_3	195.5[a] ± 10.8
	+ oPRL	187.0[a] ± 25.5
	+ Dex.	152.3 ± 13.0
	+ EGF	61.4[b] ± 5.5
Glucagon		114.5 ± 11.1
	+ T_3	105.9 ± 13.1
	+ oPRL	105.9 ± 20.8
	+ Dex.	194.9 ± 15.9
	+ EGF	68.8[b] ± 5.5
bGH + Glucagon		319.1 ± 17.4
	+ T_3	357.3 ± 15.7
	+ oPRL	392.6 ± 25.2
	+ Dex.	287.5 ± 22.7
	+ EGF	206.3[d] ± 25.2

[a] $p < 0.01$, [b] $p < 0.005$, [c] $p < 0.001$, [d] $p < 0.05$ compared to appropriate controls.

Hepatocytes were prepared and cultured as described previously (19). 72 h after plating, the cells were treated for 6 h with 10 nM T_3, 200 ng/ml oPRL, 100 nM dexamethasone or 20 ng/ml EGF in the presence or absence of bGH (50 ng/ml), glucagon (100 ng/ml) or the combination of bGH plus glucagon. Total RNA (40 ug) was subjected to Northern and dot blot analyses using a ^{32}P-labeled IGF-I cDNA probe. The probe was stripped from the membranes which were then rehybridized with a ^{32}P-labeled GAPDH cDNA probe as described in Materials and Methods. Normalized densitometric readings of dot blots are expressed as mean ± S.D. of triplicate determinations. All the values of IGF-I mRNA levels are expressed as % of control which is normalized to 100%

Table 2. Effect of insulin on IGF-I mRNA levels in the presence of T_3, dexamethasone, bGH, glucagon and their combinations

Hormonal Treatment	Insulin (100 nM)	IGF-I mmRNA levels Relative to Control
Control	-	100
Control	+	38.1[a] ± 15.1
Glucagon	-	114.5 ± 11.1
Glucagon	+	57.9[b] ± 7.8
T_3	-	68.4 ± 7.1
T_3	+	108.7[c] ± 4.2
bGH	-	132.2 ± 12.0
bGH	+	63.2[b] ± 2.3
Dex.	-	156.3 ± 24.1
Dex.	+	136.7 ± 18.8
Glucagon + bGH	-	319.1 ± 17.4
Glucagon + bGH	+	180.5[b] ± 10.6
T_3 + bGH	-	228.4 ± 41.0
T_3 + bGH	+	149.3 ± 24.1
T_3 + Dex.	-	176.2 ± 22.4
T_3 + Dex.	+	131.3 ± 14.2
Dex. + bGH	-	216.0 ± 14.8
Dex. + bGH	+	234.6 ± 7.1
T_3 + Dex. + bGH	-	280.6 ± 39.0
T_3 + Dex. + bGH	+	180.6 ± 22.4

[a] $p < 0.005$, [b] $p < 0.01$, [c] $p < 0.05$ compared to their controls containing no insulin.

Cultured hepatocytes were prepared as described previously (19). After 72 h in culture, insulin (10^{-7} M) in the presence or absence of 100 ng/ml glucagon, 10 nM T_3, 100 nM dexamethasone was added. Total RNA was extracted after 6 h of incubation and subjected to Northern and dot blot analyses as noted in Materials and Methods. IGF-I mRNA levels in all the samples are expressed as % of that in untreated cells (control). Normalized IGF-I mRNA levels (ratio of IGF-I mRNA to GAPDH mRNA) are expressed as mean ± S.D. of triplicate determinations.

mRNA levels but either had no effect or inhibited IGF-I mRNA levels in the presence of various combinations of dexamethasone, bGH and T_3.

cAMP and IBMX mimick the effect of glucagon on IGF-I mRNA levels

Hepatocytes were treated with bGH in the presence and absence of glucagon, IBMX and $(Bu)_2$cAMP (Figure 1). Without additions (control), IGF-I mRNA levels were low as noted previously (19). The addition of bGH alone stimulated IGF-I mRNA levels 2 to 3 - fold. Glucagon, IBMX and $(Bu)_2$cAMP each stimulated IGF-I mRNA levels 1.2 to 1.8 - fold but, produced in combination with bGH, a markedly augmented IGF-I mRNA levels by 10 to 12 -fold. These results suggest that glucagon produces its synergistic effect with bGH on IGF-I mRNA levels by activating PKA.

As shown in Table 3, the separate addition of bGH, bGH or $(Bu)_2$cAMP stimulated IGF-I mRNA levels 1.2 to 1.7 - fold. Their combination showed a dose-dependent synergistic stimulation of IGF-I mRNA levels upto 7 - fold control levels. This confirmed the data of Fig. 1. In the presence of bGH (50 ng/ml), increasing $(Bu)_2$cAMP from 10 to 500 ug/ml produced a marked augmentation. Thus the effect of combining bGH and $(Bu)_2$cAMP closely approximates that seen on combining bGH and glucagon (19).

Figure 1. Effect of IBMX and $(Bu)_2$cAMP in the presence and absence of bGH on IGF-I mRNA accumulation in cultured rat hepatocytes

1 A Autoradiogram of Northern blot of total RNA from rat hepatocytes. Primary cultured rat hepatocytes were prepared and maintained in a serum free medium as described previously (19). 72 h after plating, the hepatocytes were treated with no additions (lane 1), 50 ng/ml bGH (lane 2), 100 ng/ml glucagon (lane 3), 50 ng/ml bGH plus 100 ng/ml glucagon (lane 4), 100 uM IBMX (lane 5), 50 ng/ml bGH plus 100 uM IBMX (lane 6), 150 uM $(Bu)_2$cAMP (lane 7), 50 ng/ml bGH plus 150 uM $(Bu)_2$cAMP (lane 8) for 6 h. At the end of incubation period, total RNA was extracted as described in Materials and Methods. 40 ug of total RNA was subjected to Northern blot analysis using a ^{32}P-labeled IGF-I cDNA probe.

1 B The Northern blot in 1A was stripped and rehybridized with a ^{32}P-labeled GAPDH cDNA probe to normalize for possible variations in loading and transfer of RNA.

Figure 1

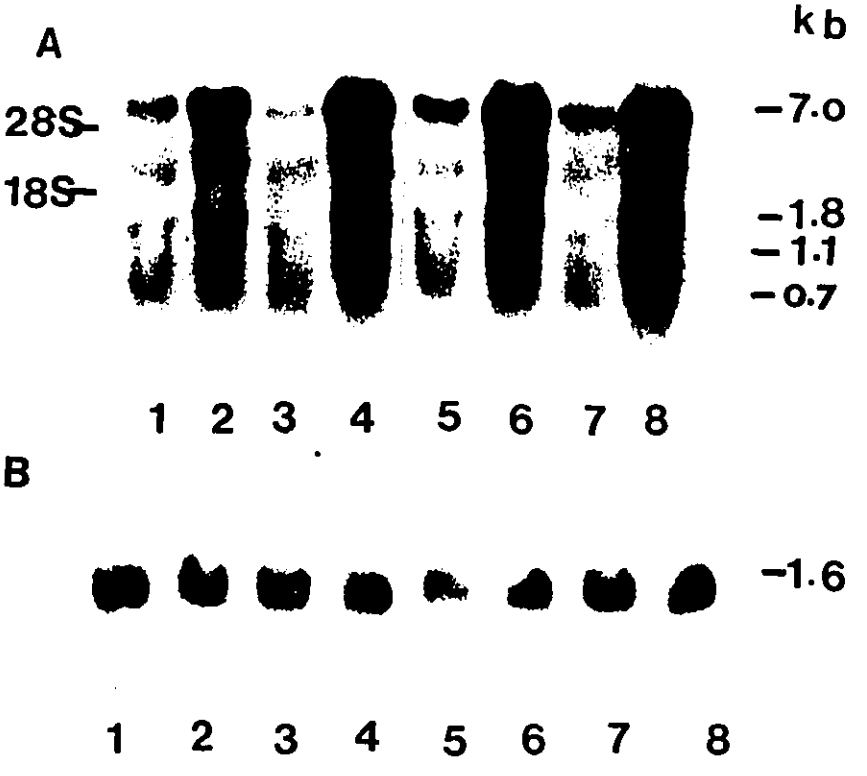


Figure 1

1 C Quantitation of IGF-I mRNA. The normalized densitometric reading of IGF-I mRNA (the ratio of IGF-I to GAPDH mRNA) are expressed as mean ± S.D. of triplicate determinations. IGF-I mRNA levels in all the samples are expressed as % of that in untreated cells (control).

*p < 0.01 compared to glucagon treated control and IBMX treated control.

**p < 0.005 compared to $(Bu)_2$cAMP treated control.

This experiment is representative of the two experiments carried out on different occasions and similar results were obtained.

Figure 1

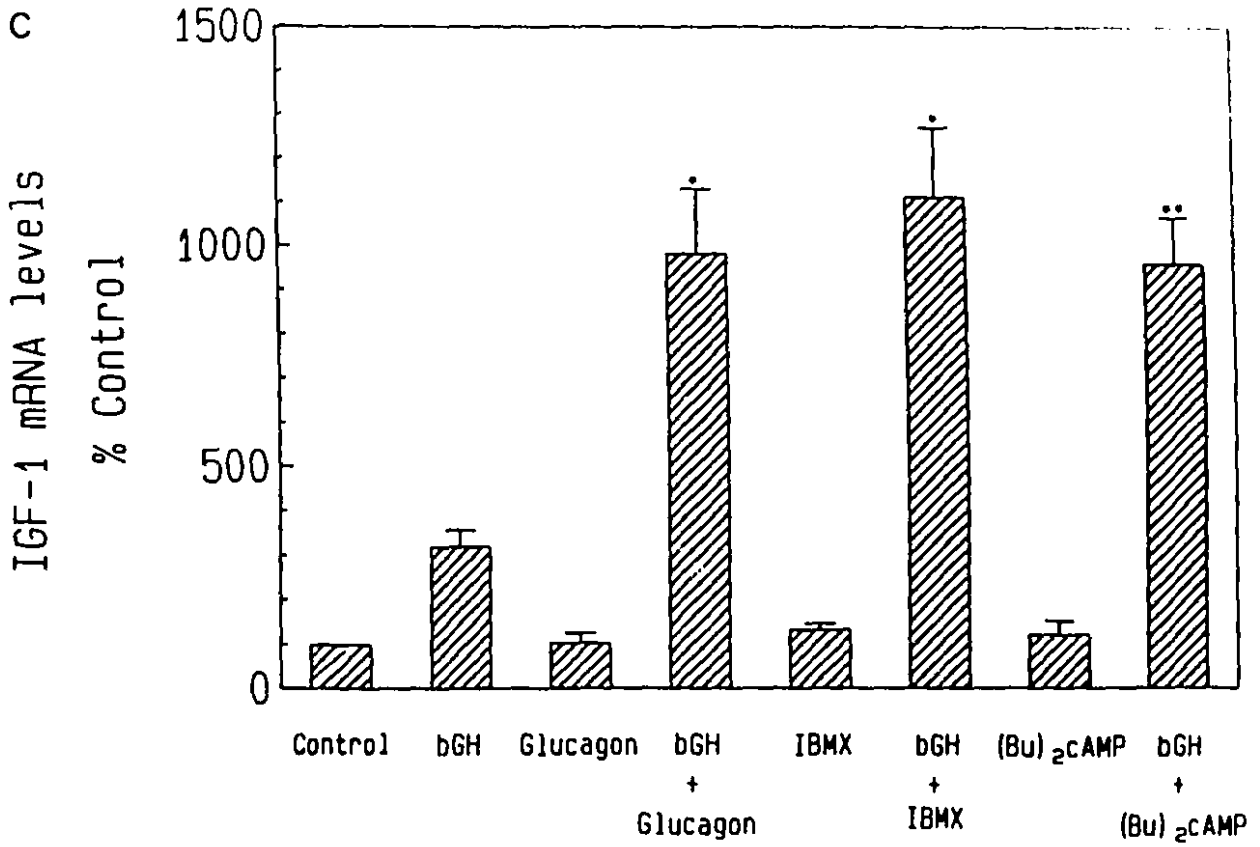

C
IGF-1 mRNA levels
% Control
1500
1000
500
0
Control
bGH
Glucagon
bGH + Glucagon
IBMX
bGH + IBMX
(Bu)2cAMP
bGH + (Bu)2cAMP

Table 3. Dose dependent effect of bGH and/or $(Bu)_2$cAMP on IGF-I mRNA levels in hepatocytes

Treatment		IGF-I mRNA levels % Control
bGH (ng/ml)	$(Bu)_2$cAMP (uM)	
10	-	121.0 ± 14.8
100	-	169.2[a] ± 9.5
300	-	141.5 ± 12.7
-	10	117.3 ± 4.3
-	100	159.2[b] ± 2.5
-	150	143.2[a] ± 6.7
50	5	151.6 ± 9.4
50	10	165.9 ± 11.9
50	50	695.7 ± 36.1
50	100	535.4 ± 34.1
50	150	529.1 ± 25.3

[a] $p < 0.005$, [b] $p < 0.001$ compared to control.

72 h after plating, the hepatocytes were treated with bGH, $(Bu)_2$cAMP or their combinations for 6 h as shown above. RNA samples from hepatocytes were subjected to Northern and dot blot analysis using IGF-I cDNA probe. The blots were stripped and rehybridized with GAPDH cDNA probe. The normalized densitometric reading of IGF-I mRNA (ratio of IGF-I mRNA to GAPDH mRNA) is expressed as mean ± S.D.of triplicate determination. The values of all the samples are expressed relative to control.

Glucagon's effect on IGF-I mRNA levels does not require PKC

In rat hepatocytes, glucagon has been shown to stimulate the production of inositol phosphate as well as that of cAMP (22). PMA was found to mimick the effect of glucagon on hepatic steroid metabolism (23). To evaluate the involvement of protein kinase C (PKC) in regulating hepatic IGF-I mRNA levels, cells were treated with PMA and bGH plus PMA. As shown in Table 4, PMA minimally stimulated IGF-I mRNA levels 1.2 to 1.3 - fold whereas the inactive analog (4α-phorbol 12, 13, didecanoate) had no effect. PMA plus bGH only stimulated IGF-I mRNA levels 1.6 to 1.8 - fold. These results indicate that the effects of glucagon does not involve the activation of PKC.

Effect of preincubation with PMA on the stimulation of IGF-I mRNA levels by bGH and glucagon

It has been previously observed that GH promotes increased DAG production (20,24,25,33) and activates PKC (26,34) in hepatocytes and other cell types. Prolonged treatment with PMA has been shown to reduce the cellular levels of PKC (35,36). In order to test whether the observed effects of bGH plus glucagon require PKC activation, cells were depleted of PKC by preincubation with 10 uM PMA for 24 h. As shown in Figure 2, preincubation with PMA, markedly inhibited the effects of bGH plus glucagon (80% inhibition), bGH alone (40% inhibition) and PMA itself (60% inhibition) on IGF-I mRNA levels in cultured hepatocytes. The effect of glucagon alone was not significantly inhibited by preincubation with PMA, an observation consistent

Table 4. Effect of PMA in the presence or absence of bGH on IGF-I mRNA levels

Treatment	IGF-I mRNA levels % Control
bGH	242.2[a] ± 28.2
Glucagon	111.4 ± 18.1
PMA	128.3[b] ± 8.1
α-PDco$_2$	103.0 ± 13.5
bGH + Glucagon	841.6[a] ± 42.8
bGH + PMA	177.9 ± 13.8

[a] $p < 0.005$, [b] $p < 0.01$

72 h after plating, the hepatocytes were treated with bGH (50 ng/ml), glucagon (100 ng/ml), PMA (100nM) or α-PDco$_2$ (100 nM) alone or in combination for 6 h. Total hepatocyte RNA was extracted subjected to electrophoresis and dot blot analyses as noted in Materials and Methods. Normalized IGF-I mRNA levels (ratio of IGF-I to GAPDH mRNA) are expressed as mean ± S.D. of triplicate detreminations. IGF-I mRNA levels are expressed as a % of that in untreated (control) cells.

Figure 2. Effect of bGH and/or glucagon on IGF-I mRNA levels with and without prior preincubation with PMA

2 A. Autoradiogram of Northern blot of total RNA from hepatocytes. Cultured hepatocytes were prepared as described previously (19). 72 h after plating, the cells were either preincubated with 10 uM PMA or with vehicle (-PMA) for 24 h. The cells were rinsed three times with a serum free medium and further incubated with no additions (lanes 1), 50 ng/ml bGH (lanes 2), 100 ng/ml glucagon (lanes 3), 50 ng/ml bGH plus 100 ng/ml glucagon (lanes 4) and 100 nM PMA (lanes 5) for 6 h. 40 ug of total RNA samples were subjected to Northern blot analyses as described in Materials and Methods.

2 B. To normalize IGF-I mRNA levels, the Northern blot (2A) was stripped and rehybridized with a ^{32}P-labeled GAPDH probe.

Figure 2

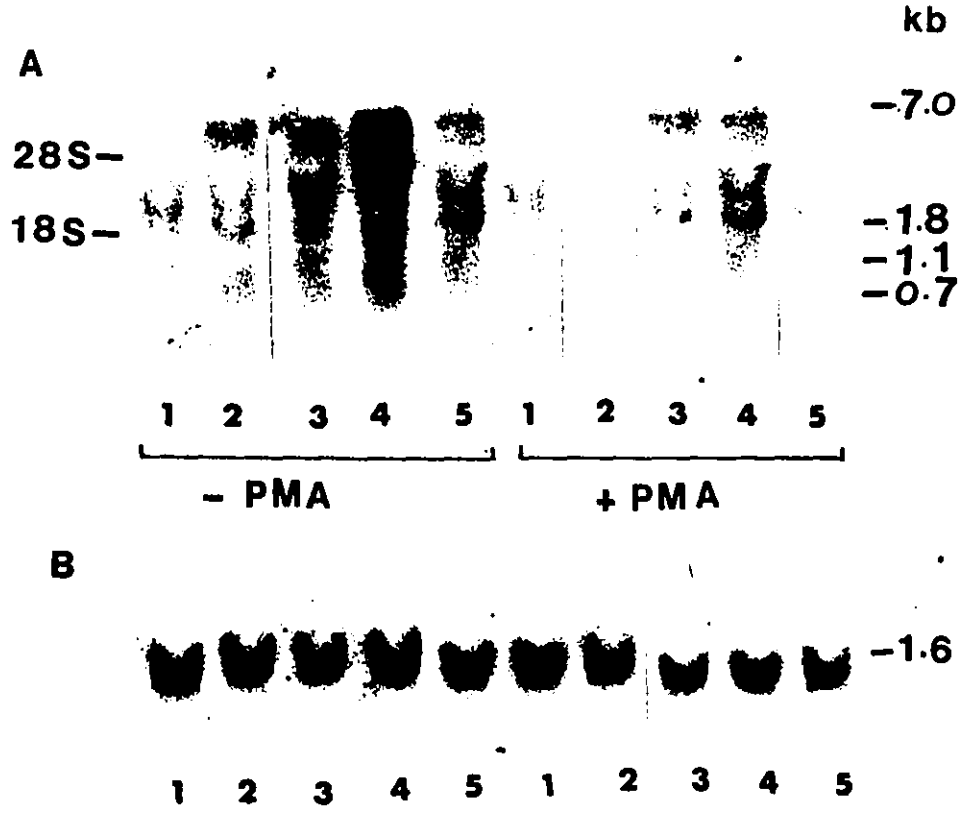
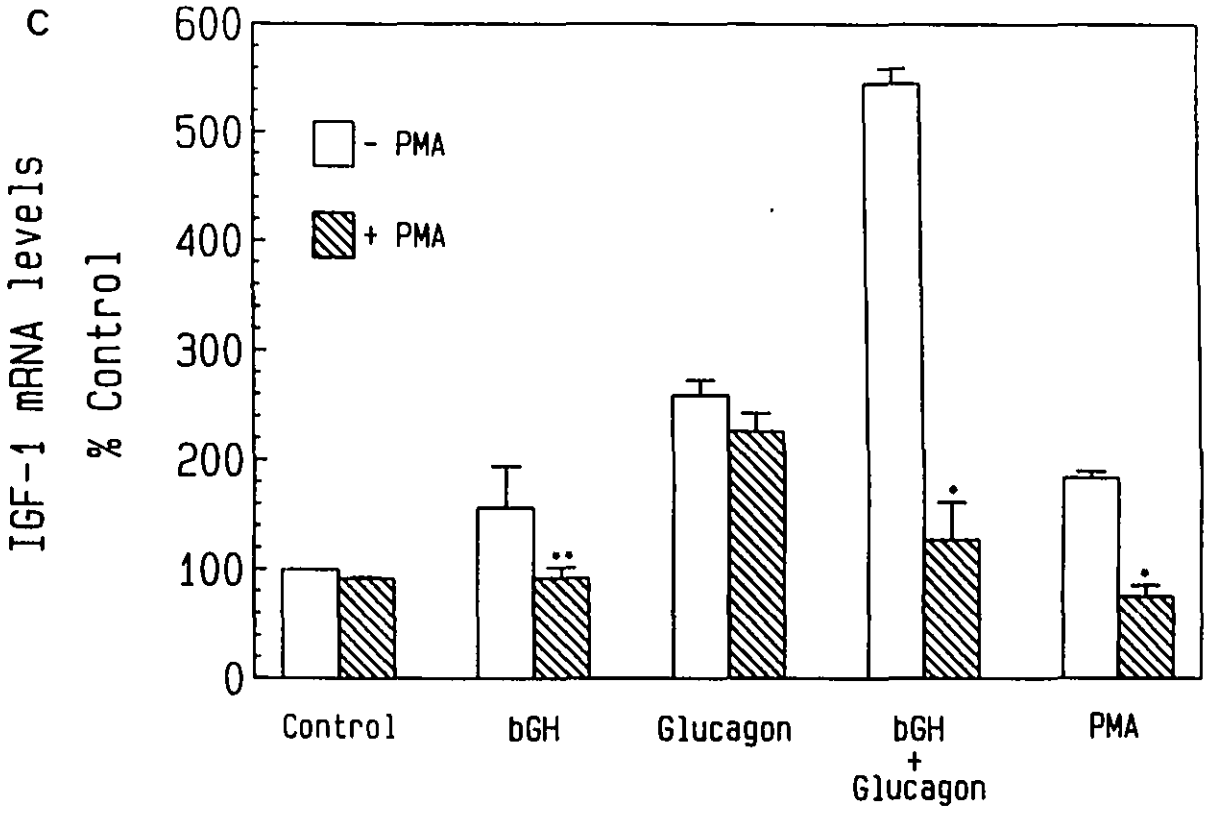


Figure 2

2 C. Quantitation of IGF-I mRNA. Normalized densitometric reading of IGF-I mRNA (the ratio of IGF-I mRNA to GADHP mRNA) is expressed as mean ± S.D. of triplicate determinations of dot blots.

◆$p < 0.001$, ✱$p < 0.01$, ✱✱$p < 0.05$ compared to their controls.

Figure 2


C
IGF-1 mRNA levels
% Control
600
500
400
300
200
100
0
- PMA
+ PMA
Control
bGH
Glucagon
bGH
+
Glucagon
PMA

with that of Table 4, indicating that glucagon does not produce its effects via PKC activation. On the other hand GH action is clearly blunted by preincubation with PMA indicating a requirement for PKC activation to effect the GH response.

Further evidence of involvement of protein kinases on bGH and/or glucagon effect on IGF-I mRNA levels

In order to substantiate our previous results, inhibitors of PKC (35-41) and other protein kinases (38,40,41) were evaluated. Figure 3 shows IGF-I mRNA levels in cells pretreated with staurosporine (39), sphingosine (37,38) or H7 (40,41). The effect of bGH was reduced by 40-50%, glucagon by 20-25% and bGH plus glucagon by 80-90%. The partial inhibition of glucagon's stimulatory effect by sphingosine and H_7 is consistent with prior observations demonstrating that these agents are not specific for PKC but inhibit PKA activity as well (38,41).

Effect of PMA plus glucagon on IGF-I mRNA levels

As shown previously (Figs. 2 and 3) bGH appears to stimulate IGF-I mRNA levels via PKC activation. To see if stimulating PKC and PKA is sufficient to augment IGF-I mRNA levels, hepatocytes were treated with PMA plus glucagon as shown in Figure 4. PMA plus glucagon showed no synergism on IGF-I mRNA levels.

As shown in Table 5, PMA stimulated IGF-I mRNA levels 1.2 to 1.4 fold. Various concentrations of $(Bu)_2$cAMP (5-500uM) in the presence of 50 nM PMA stimulated IGF-I mRNA levels 1.2 to

Figure 3. Effect of inhibitors of protein kinases on bGH and/or glucagon stimulated IGF-I mRNA levels

IGF-I mRNA levels in hepatocytes. Cultured rat hepatocytes were prepared as described previously (19). 72 h after plating, the cells were treated with 500 nM staurosporine, 100 uM sphingosine or 1 mM H_7, 30 min prior to the addition 50 ng/ml bGH, 100 ng/ml glucagon and 50 ng/ml bGH plus 100 ng/ml glucagon for 6 h. The inhibitors were present throughout the incubation period. Total RNA samples prepared from the hepatocytes were subjected to Northern and dot blot analyses. Normalized densitometric reading (the ratio of IGF-I to GAPDH mRNA) are expressed as mean ± S.D. of triplicate determinations of dot blots. The values of IGF-I mRNA levels are expressed relative to that of untreated cells (control).

♦$p < 0.001$, ♦♦$p < 0.005$, ✱$p < 0.01$ compared to appropriate controls.

Figure 3

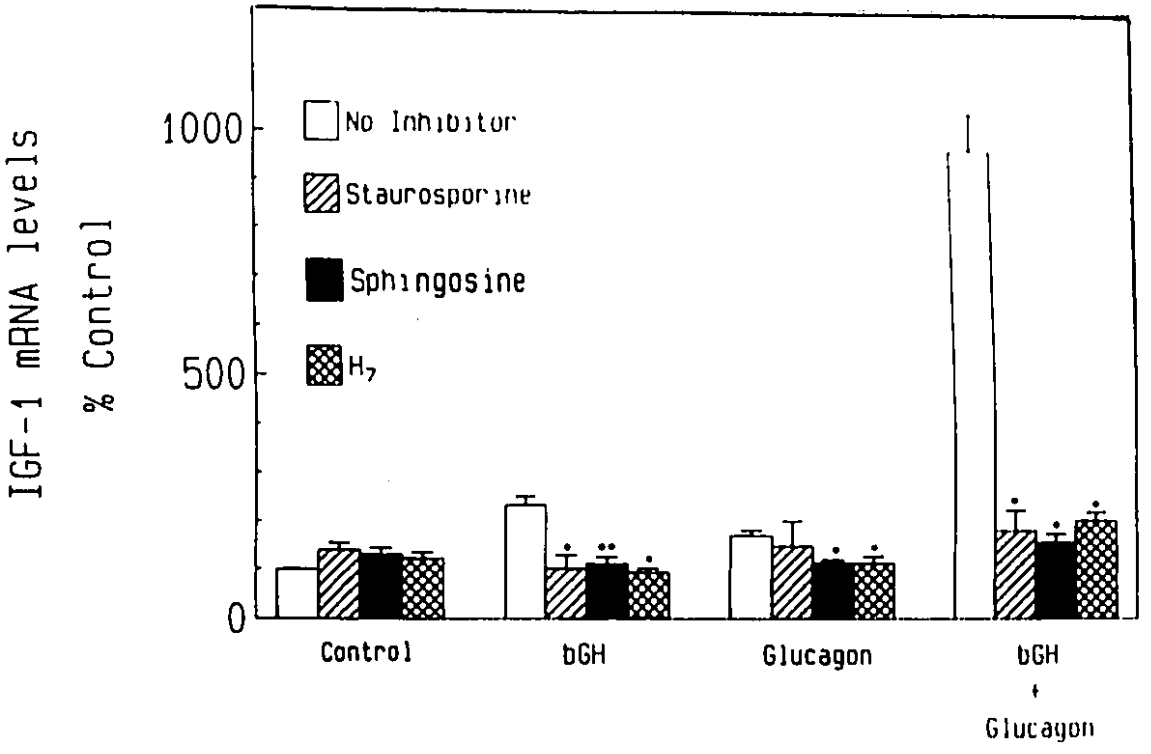

IGF-1 mRNA levels
% Control
1000
500
0
No Inhibitor
Staurosporine
Sphingosine
H7
Control
bGH
Glucagon
bGH
+
Glucagon

Figure 4. Effect of activation of PKC in the presence of glucagon on IGF-I mRNA accumulation in hepatocytes

IGF-I mRNA levels in hepatocytes. Cultured rat hepatocytes were prepared as described previously (19). 72 h after plating, the cells were treated with 50 ng/ml bGH, 100 ng/ml glucagon, 50 ng/ml bGH plus 100 ng/ml glucagon, 100 nM PMA and 100 nM PMA plus 100 ng/ml glucagon for 6 h. Total RNA samples were subjected to Northern and dot blot analyses as described in Materials and Methods. Normalized IGF-I mRNA levels (the ratio of IGF-I to GAPDH mRNA) is expressed as mean ± S.D. of triplicate determinations of dot blots. The values of all the samples are expressed relative to that of untreated control.

Figure 4

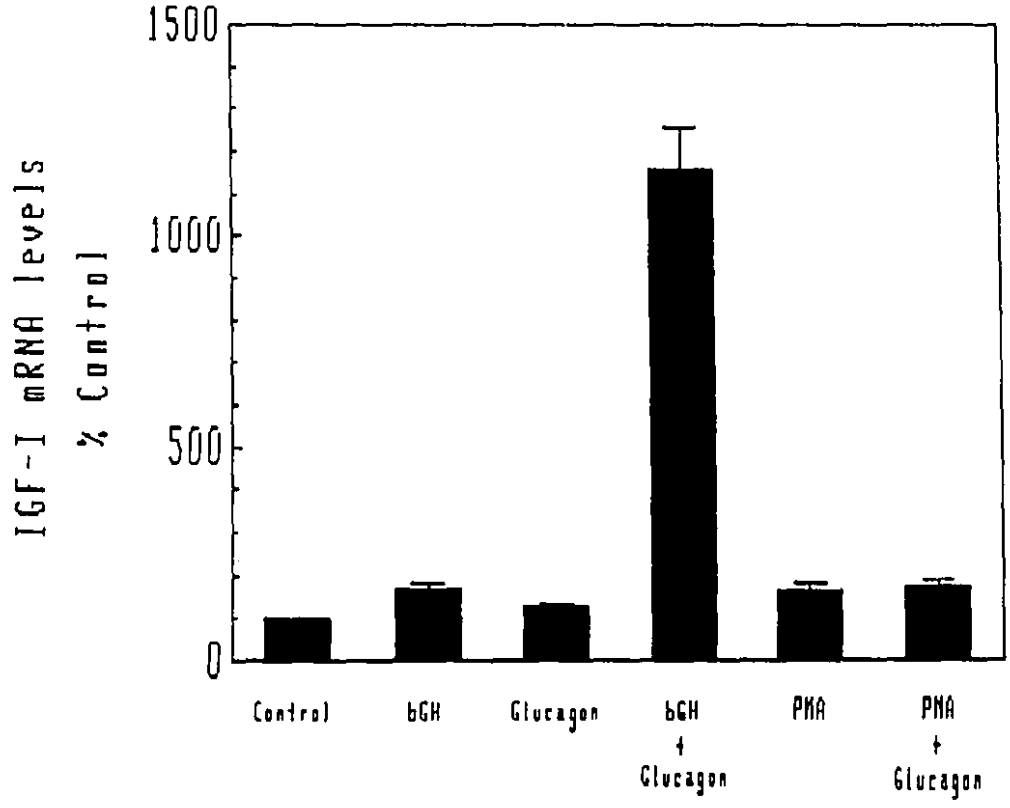

IGF-I mRNA levels
% Control
1500
1000
500
0
Control
bGH
Glucagon
bGH + Glucagon
PMA
PMA + Glucagon

Table 5. Dose dependent effect of PMA and PMA plus $(Bu)_2$cAMP on IGF-I mRNA levels

Treatment		IGF-I mRNA levels % Control
PMA (nM)	$(Bu)_2$cAMP (uM)	
5	-	121.1 ± 8.7
50	-	131.1 ± 10.5
500	-	133.9[a] ± 13.2
50	5	110.7 ± 18.4
50	50	128.7 ± 9.7
50	500	151.9[b] ± 17.8

[a] $p < 0.01$, [b] $p < 0.005$

Hepatocytes were cultured, RNA was extracted and IGF-I mRNA levels were determined as described in legend of Table 1. The values are the mean ± S.D. of triplicated determinations.

1.7 fold. These results are consistent with those obtained in Figure 4. Therefore activation of both PKC and PKA is necessary but not sufficient to produce the synergistic response observed with bGH plus glucagon.

DISCUSSION

The effect of GH on hepatic IGF-I mRNA levels has been documented both in vivo (5,7,10) and in vitro (17,42). The mechanism by which GH augments IGF-I mRNA levels has not been defined. We previously showed that bGH plus gulcagon synergized to stimulate IGF-I mRNA levels 10 to 12 fold in rat hepatocytes (17). T_3, oPRL, dexamethasone, EGF and insulin showed no synergistic effect on stimulation of IGF-I mRNA levels when added in combination with bGH or glucagon. This confirmed the unique role of bGH and glucagon on stimulation of IGF-I mRNA levels in hepatocytes. T_3 has been shown to have no effect of its own but potentiates GH effect on IGF-I mRNA levels in vivo (9) and in vitro (42). T_3 plus bGH showed 4.0 fold stimulation (42) compared to 1.5 fold stimulation we have observed on IGF-I mRNA levels in rat hepatocytes. This discrepancy could be explained by different culture conditions and the presence of insulin in the culture medium (42), as insulin has been shown to stimulate GH binding (42). The stimulatory effect of bGH plus T_3 on IGF-I mRNA levels could be due to increased GH binding in the presence of T_3. GH binding increases in hyperthyroid rat liver membrane and decreases in hypothyroid rats (43).

Our results indicate that oPRL has no effect on IGF-I mRNA levels in the presence or absence of glucagon, whereas in the presence of bGH or bGH plus glucagon, it stimulates IGF-I mRNA levels 1.4 to 1.5 fold. Since oPRL does not synergize with

glucagon to stimulate IGF-I mRNA levels, oPRL appears to act via its own receptors. Although oPRL has been shown to stimulate IGF-I mRNA levels in vivo in hypophysectomized rat through its somatogenic activity (44).

Here we report that dexamethasone in the pesence or absence of bGH showed no stimulatory or inhibitory effect on IGF-I mRNA accumulation. This is contrary to the inhibitory effect of dexamethasone shown on hepatic IGF-I mRNA levels in vivo in hypophysectomized rat before and after GH therapy (45) and also in vitro in rat neuronal and glial cells in primary culture (46). The inhibitory effect of dexamethasone on IGF-I mRNA levels in vivo (45) could be due to the interactions of various hormones, whereas its effect in vitro (46) may depend on the culture conditions and the cell types used. To our knowldge, the effect of EGF on IGF-I mRNA levels has not been previously shown. Our data show the inhibitory effect of EGF on IGF-I mRNA levels in the presence or absence of bGH, glucagon or bGH plus glucagon.

Insulin inhibits IGF-I mRNA levels by 40 to 60% in the presence or absence of bGH, glucagon or bGH plus glucagon. Johnson et al (31) and Boni-Schnetzler et al (32) showed stimulatory effect of insulin on IGF-I mRNA levels in hepatocytes. Johnson et al (31) used T_3, dexamethasone and insulin in culture medium and treated the cells with insulin for 24 h. Boni-Schnetzler et al (32) used dexamethasone and insulin in the culture medium and showed stimulation of IGF-I

mRNA levels in rat hepatocytes after 16 h of incubation with insulin. In both cases, experiments were carried out after 24 h of initiating the culture. Tollet et al (42) showed no effect of insulin on IGF-I mRNA levels but reported potentiating effect of insulin on GH induction of IGF-I mRNA accumulation in rat hepatocytes. Again insulin was present in the culture medium and the cells were treated with various hormones for 24 h. The discrepancy between these results and the results we have reported about the effect of insulin could be because T_3, dexamethasone and insulin were not present in our culture medium and also the cells were treated with insulin for 6 h only. The hapatocytes were maintained in culture for 72 h before carrying out the experiment as this was found to be essential to obtain a maximum response of bGH plus glucagon on IGF-I mRNA levels in hepatocytes (data not shown). It appears that the effect of insulin is an indirect effect on IGF-I mRNA levels and the cells have to be incubated with insulin in the presence of T_3, dexamethasone or bGH for a longer period of time. Dexamethasone, glucagon and $(Bu)_2$cAMP have been shown to up regulate GH receptors whereas EGF and insulin have been shown to down regulate GH receptors in hepatocytes (47). Insulin stimulates GH binding in hepatocytes (42). The stimulation or inhibition of IGF-I mRNA levels seen with various hormones or agents in hepatocytes in the presence of GH is due to an increased number of GH receptors or increased binding of GH to its receptors need further investigation.

Since glucagon and GH appear to be the major hormonal

regulators of hepatic IGF-I mRNA levels, the mechanism of their effects on stimulation of IGF-I mRNA levels were investigated. Glucagon has been shown to stimulate the production of inositol phosphate as well as that of cAMP in rat hepatocytes (22). We observed previously unrecognized effect of $(Bu)_2$cAMP or IBMX in the presence of bGH to produce a synergistic effect on IGF-I mRNA levels. $(Bu)_2$cAMP (5-150uM) alone stimulated IGF-I mRNA levels 1.4-1.6 fold but synergized wth bGH (50 ng/ml) in a dose dependent manner to stimulate IGF-I mRNA levels 5.0 to 8.0 fold.

The stimulatory effect of cAMP on IGF-I mRNA levels has been reported in rat hepatocytes (33) and osteoblast enriched cultures (48). Tollet et al (33) showed a stimulatory effect of glucagon but failed to observe synergistic effect of glucagon plus GH or cAMP plus GH on IGF-I mRNA levels in rat hepatocytes. Again this could be due to different culture conditions employed.

To determine if glucagon also acted via PKC pathway to stimulate IGF-I mRNA levels, hepatocytes were treated with bGH plus PMA. IGF-I mRNA levels were stimulated 1.7 to 1.8 fold by bGH plus PMA compared to 8.0 to 9.0 fold stimulation observed by bGH plus glucagon, whereas 4α-phorbol 12,13-didecanoate (α-$PDco_2$), inactive in PKC modulation had no effect on IGF-I mRNA levels.

Though a small stimulation of IGF-I mRNA is observed by elevating PKC levels, no synergism was observed by combining

PMA and bGH. These observations indicate that glucagon's effect on IGF-I mRNA levels in the presence of bGH appears to be via PKA pathway rather than by elevation of PKC levels. We cannot rule out the possibility that PMA may not be able to stimulate all the species of PKC in hepatocytes (36).

We explored the mechanism of GH effect on IGF-I mRNA stimulation. As GH has been documented in exerting some of its effects via elevating PKC levels (24,25,26,33,34), we investigated if GH effect on stimulation of IGF-I mRNA levels was via elevation of PKC levels. bGH plus glucagon effect on IGF-I mRNA level was inhibited 80% in PKC depleted cells, bGH and PMA effects were inhibited 40% and 60% respectively, whereas glucagon's effect was unaltered. It is possible that PMA may not down regulate all the species of PKC at the same rate (36). In the light of the results obtained in PKC depleted cells, the effect of inhibitors of PKC were investigated. Staurosporine (39), sphingosine (37,38) and H7 (40,41) were found to inhibit bGH effect 50-60% and bGH plus glucagon effect 80-90%. Sphingosine and H7 inhibited glucagon effect 20-30% consistent with previous observations that the above inhibitors are nonspecific and tend to inhibit other protein kinases such as PKA besides PKC. Tollet et al (33) demonstrated a role for protein kinase C in GH stimulation of IGF-I mRNA in rat hepatocytes. Though bGH appears to act via PKC pathway and glucagon via elevation of PKA to produce a synergistic effect on stimulation of IGF-I mRNA levels, PMA plus glucagon or PMA plus $(Bu)_2$cAMP do not appear to synergize IGF-I mRNA levels.

This suggests that bGH may stimulate or modify protein(s) or transcription factor(s) besides elevating PKC levels which may be essential for producing a synerigstic effect on IGF-I mRNA levels in the presence of PKC and PKA.

GH has been shown to stimulate tyrosine kinase activity associated with GH receptor in various cell types (49) and also tyrosyl phosphorylation of cellular proteins (50). There may a possible role of tyrosine kinase activity in action of GH. GH has also been shown to stimulate c-jun and c-fos mRNAs in 3T3-F442A preadipocytes (34). Phosphorylated jun/fos heterodimer stimulated by phorbol ester binds and stimulates transcription from the AP-1 enhancer sequence. AP-1, AP-2 and AP-3 are recognized by phorbol esters modulated transacting factors (51).

Our results show that cAMP and bGH produce a synergistic effect on stimulation on IGF-I mRNA levels. If the effect of cAMP is at the level of gene transcription, there has to be either cAMP regulatory element (CRE), AP-1 or AP-2 binding sites (52) on IGF-I gene promoter. Two promoters for human (53) and rat (54) IGF-I gene have been identified and a promoter for exon 1 from human has been characterized (53). But the available data show no evidence for the presence of these sites on IGF-I gene. AP-1 or AP-2 transcription factors also mediate the actions of PKC on gene expression (55,56), although published evidence indicates that signaling through this kinase enhances IGF-I gene transcription in U 937 macrophage cell line

(57). Kim S-W et al (53) reported no sequence in human IGF-I promoter or exon 1 that resembles the putative GH reponse element recently identified by Yoon et al in Spi 2.1 gene promoter (58). Perhaps these cis acting elements are found elsewhere in IGF-I gene. There is a potential recognition sites for AP-1/jun (51) in chicken IGF-I gene promoter (59).

Since promoters for rat IGF-I gene have not been characterized (54), it is not possible to explain the mechanism of GH or cAMP effect on IGF-I gene transcription. GH has been shown to stimulate IGF-I gene transcription (7), but whether cAMP acts at a transcriptional level of IGF-I gene is not known. More work is required to determine if the effect of cAMP on IGF-I mRNA accumulation is at the level of transcription or is posttranscriptional. Whether the effect of various hormones or reagents observed on IGF-I mRNA levels correlate with actual synthesis of IGF-I peptide need further investigation.

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CHAPTER 5

A NEW PROTEIN(S) SYNTHESIS IS REQUIRED FOR BOVINE GROWTH HORMONE AND GLUCAGON EFFECT ON IGF-I mRNA STIMULATION IN RAT HEPATOCYTES

PREFACE TO CHAPTER 5

The mechanisms of bGH and glucagon effect on IGF-I mRNA stimulation are futher investigated in this chapter.

As discussed in chapter 4, bGH and glucagon were found to stimulated IGF-I mRNA levels via PKC and PKA pathways respectively. But elevation of PKC and PKA levels failed to produce a synergistic effect on the stimulation of IGF-I mRNA levels. These data indicate that stimulation of PKA and PKC are necessary but not sufficient to produce a synergistic effect on IGF-I mRNA stimulation. To determine if a new protein synthesis was required besides elevation of PKC and PKA levels to produce a synergistic effect on IGF-I mRNA levels, cycloheximide was used to inhibit protein synthesis by 92 %. The presence of cycloheximide inhibited bGH plus glucagon's synergistic effect on IGF-I mRNA levels by 85 %. These data indicate that the synergistic effect of bGH plus glucagon on IGF-I mRNA stimulation requires synthesis of a new protein besides elevation of PKC as well as PKA. bGH was found to stimulate IGF-I mRNA levels at the level of gene transcription whereas glucagon's effect was partly at a posttranscriptional level to increase IGF-I mRNA stability.

ABSTRACT

The goal of this study was to investigate the mechanism underlying the synergistic effect of growth hormone and glucagon on IGF-I mRNA stimulation in rat hepatocytes. Earlier work from our laboratory showed that bGH (50 ng/ml) plus glucagon (100 ng/ml) synergized to stimulate IGF-I mRNA levels 10 to 12 fold in rat hepatocytes [Kachra et al (1991) Endocrinology 128:1723-1730]. This effect of glucagon was mimicked by 0.1 mM IBMX or 0.1 mM $(Bu)_2$cAMP (manuscript in preparation). The half life of IGF-I mRNA determined by decay rate in the presence of 5 ug/ml actinomycin D (sufficient to inhibit RNA synthesis by 92%) was 12 h. The effect of 50 ng/ml bGH, 100 ng/ml glucagon and 0.1 mM $(Bu)_2$cAMP treatment on mRNA stability were assessed by decay after inhibition of RNA synthesis with actinomycin D. bGH had no significant effect on IGF-I mRNA stability whereas glucagon or $(Bu)_2$cAMP in the presence or absence of bGH appeared to stabilize IGF-I mRNA 75-94% of the control at 0 time after 10 h of incubation. 10 ug/ml cycloheximide, which was sufficient to inhibit protein synthesis by 92%, blocked bGH plus glucagon effect by 75-80% and bGH plus $(Bu)_2$cAMP effect by 60-65% on stimulation of IGF-I mRNA levels.

In summary glucagon and $(Bu)_2$cAMP appear to act in part at a posttraanscriptional level to stabilize IGF-I mRNA. Augmentation of IGF-I mRNA levels by bGH plus glucagon or bGH plus $(Bu)_2$cAMP appear to require synthesis of one or more

protein(s).

INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a mitogenic polypeptide hormone with structural similarities to proinsulin. It plays a fundamental role in postnatal and adolescent growth (1). IGF-I mRNA expressed in various tissues (2-5) and cultured cells (6-8) have been shown to be GH dependent. GH effect on hepatic IGF-I gene transcription has been reported in vivo by Mathews et al (8). Here we have shown that GH has no posttranscriptional effect on IGF-I mRNA stability in cultured rat hepatocytes. Earlier work from our laboratory showed that agents that elevate intracellular levels of cAMP produce a synergistic effect on stimulation of IGF-I mRNA levels when added in combination with bGH (manuscript in preparation). Whether this effect of glucagon, IBMX or $(Bu)_2$cAMP in the presence or absence of bGH on IGF-I mRNA stimulation is at the level of transcription and/or posttranscriptional is not known.

cAMP activates phosphorylating enzyme protein kinase A leading to the phosphorylation and resultant activation of transcription factor, cyclic AMP response element binding protein (CREB). Activated CREB recognizes specific DNA sequence, 5'-TGAGTCA-3', cAMP responsive enhancer (CRE) and initiates gene transcription (9). Phosphorylated CREB could also bind to AP-1 site and activate the gene promoter (10). There is no evidence for the presence of AP-1 or CRE sites on human IGF-I gene promoter for exon 1 (11). These sites may be

present elsewhere on IGF-I gene or on promoter for exon 2 which has not been characterized (11).

We previously showed that bGH appeared to act via elevation of PKC levels whereas glucagon acted via activation of PKA levels to produce a synergistic effect on IGF-I mRNA stimulation (manuscript in preparation). Elevation of PKA and PKC levels in hepatocytes showed no synergistic effect on IGF-I mRNA stimulation. This data indicate that there may be a requirement for activation or synthesis of protein(s) or factor(s) besides activation of PKC and PKA to produce a synergistic effect on IGF-I mRNA stimulation.

In the present study we have attempted to address (a) whether bGH, glucagon, bGH plus glucagon and bGH plus $(Bu)_2$cAMP act in part at a posttranscriptional level to stabilize IGF-I mRNA, (b) whether a new protein synthesis is required for bGH plus glucagon or bGH plus $(Bu)_2$cAMP effect on stimulation of IGF-I mRNA levels.

Materials and Methods

Hormones and Chemicals

Bovine growth hormone (USDA-bGH-B-1, 1.4 IU/mg) was kindly supplied by USDA Animal Hormone Program (Beltsville, MD). cycloheximide, actinomycin D, glucagon and N^6,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt were obtained from Sigma Chemical Co. (St. Louis, Mo). ^{3}H-leucine and ^{3}H-uridine were purchased from Amersham Co. (Arlington Heights IL). The chemicals and reagents used for liver perfusion, hepatocyte cultures, RNA extraction and Northern blot analysis were obtained as indicated previously (7).

Hepatocyte cultures and hormonal stimulation

Hepatocytes were isolated from male Sprague Dawley rats by perfusion in situ with collagenase (13) and maintained in a serum free medium as described previously (7). 72 h after plating, the hepatocytes were stimulated with various hormones, reagents or their combinations for 6 h.

RNA extraction, Northern and Dot blot analyses

Total RNA was extracted from the hepatocytes by guanidinium isothiocyanate cesium chloride technique (14,15) with slight modifications (7). 40 ug of total RNA was size fractionated on 1.5% agarose, 2.2 M formaldehyde denaturing gel and transfered to a hybond-N nylon membrane by capillary transfer as described

previously (7). Dot blot analyses was carried out on 5 ug of total RNA in triplicate in a Dot blot manifold from Bio-Rad using the manufacturer's protocol. RNA was fixed to the membranes by heating in an oven at 80 C for 2 h. The membranes were first hybridized with 2 x 10^6 cpm/ml of ^{32}P-labeled IGF-I cDNA probe (422 bp). To normalize the IGF-I mRNA levels, the membranes were stripped and rehybridized with 0.5 x 10^6 cpm/ml of ^{32}P-labeled GAPDH cDNA probe (750 bp). The membranes were stripped using the manufacturer's protocol for hybond-N membrane. The cDNA probes were labeled to a specific activity of 10^9 dpm/ug DNA using random primer DNA labeling system. The hybridization and washing conditions for the membranes were as described before (7).

Densitometric quantitation

RNA samples in each experiment were analysed by Northern blot as well as by Dot blot analyses in triplicate. IGF-I mRNA levels in the Northern and Dot blots were quantitated by scanning all the transcripts in an LKB Ultrascan XL enhanced laser densitometer. The results are expressed as mean ± S.D. of triplicate determination of the ratio of IGF-I mRNA to GAPDH mRNA levels of Dot blots. The levels of IGF-I mRNA in all the samples are expressed relative to control which is normalized to 100%.

Statistical analyses

Statistical analyses were carried out by paired t-test. A value of $p < 0.05$ was considered significant.

RESULTS

Inhibition of RNA synthesis

Hepatocytes were incubated with 5 ug/ml actinomycin D for 6 h to inhibit RNA synthesis. As shown Table 1, total RNA synthesis measured by ^{3}H-uridine incorporation into nucleic acids was inhibited by 92% compared to cells treated without actinomycin D. This dose of actinomycin D was used in subsequent experiments to inhibit RNA synthesis.

Half-life of IGF-I mRNA in hepatocytes

The expression of IGF-I mRNA in cultured rat hepatocyte after 72 h of incubation under serum free conditions is significantly low (7). In order to measure the decay rate over a 24 h period, IGF-I mRNA levels were stimulated by incubating the cells with 50 ng/ml bGH plus 100 ng/ml glucagon for 4 h. After removing bGH and glucagon by rinsing the cells in a serum free medium, the cells were incubated in the presence of 5 ug/ml actinomycin D over a 24 h period. As indicated in Figure 1 A and C, half-life of IGF-I mRNA is 12 h. Viability of the cells is reduced after 12 h of incubation with actinomycin D.

Posttranscriptional effect of glucagon and $(Bu)_2$cAMP

IGF-I mRNA levels in hepatocytes were stimulated by incubating the cells with 50 ng/ml bGH and 100 ng/ml glucagon for 4 h. After removing the hormones, and rinsing the cells

Table 1. Effect of actinomycin D on ^{3}H-uridine incorporation into RNA in cultured rat hepatocytes.

Treatment	^{3}H-uridine incorporation	% RNA synthesis	% inhibition of RNA synthesis
Control	5354.76 ± 904.04	100	0
Act.D	418.76 ± 16.40	7.80	92.20

Hepatocytes were plated in 20 mm six well plates and maintained in culture as described previously (7). After 72 h of plating, the cells were incubated with 5 ug/ml of actinomycin D for 6 h. The cells were pulsed with 1 uci/ml of ^{3}H-uridine 1 h before harvesting (after 5 h of incubation with actinomycin D). The proteins and the nucleic acids from the cells were precipitated with 10% TCA + 1% sodium pyrophosphate and ^{3}H-uridine incorporation was determined by counting the pellet in a scintillation counter.

Figure 1. Half-life of IGF-I mRNA in cultured rat hepatocytes.

1 A. Autoradiogram of Northern blot of IGF-I mRNA. 72 h after plating, IGF-I mRNA levels in hepatocytes were stimulated by incubating the cells with 50 ng/ml bGH plus 100 ng/ml glucagon for 4 h. The hormones were removed by rinsing the cells with serum free medium three times. The cells were incubated in a fresh medium containing 5 ug/ml actinomycin D and the decay rate of IGF-I mRNA was measured after 0 time (lanes 1,2). 1 h (lanes 3,4), 3 h (lanes 5,6), 6 h (lanes 7,8), 12 h (lanes 9,10) and 24 h (lane 11) of incubation. All the determination were made in duplicate except 24 h determination as the viability of the cells was reduced after 12 h of incubation in the presence of actinomycin D.

1 B. Northern blot A was stripped and rehybridized with GAPDH cDNA probe to normalize for possible variations in loading and transfer of RNA.

Figure 1

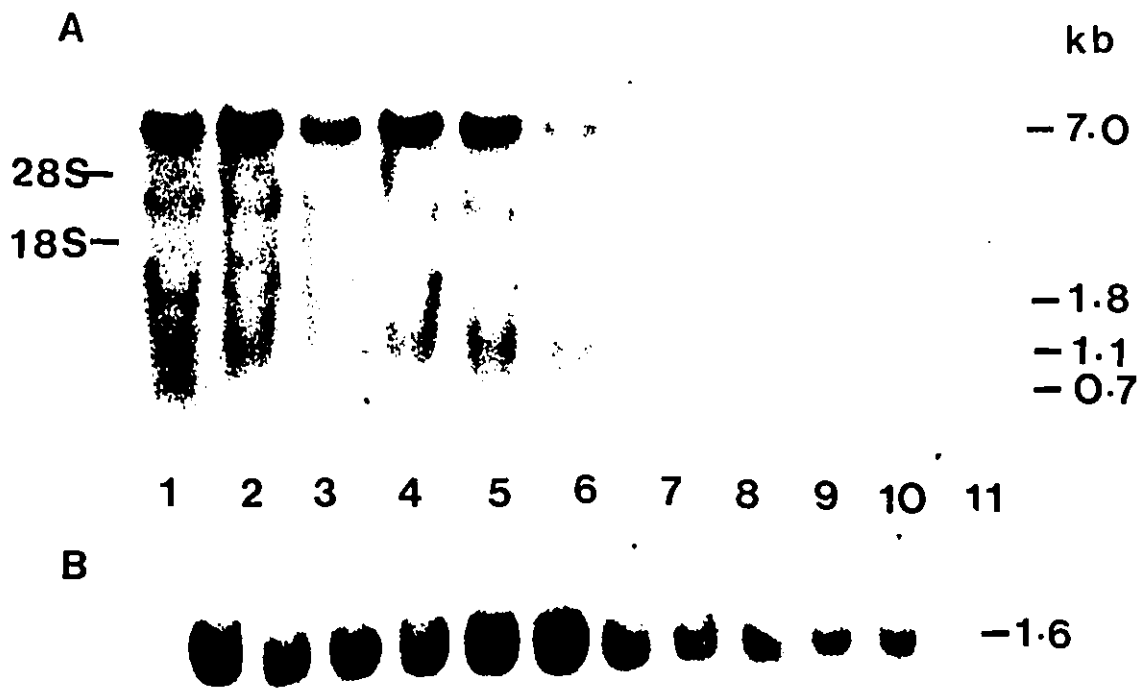
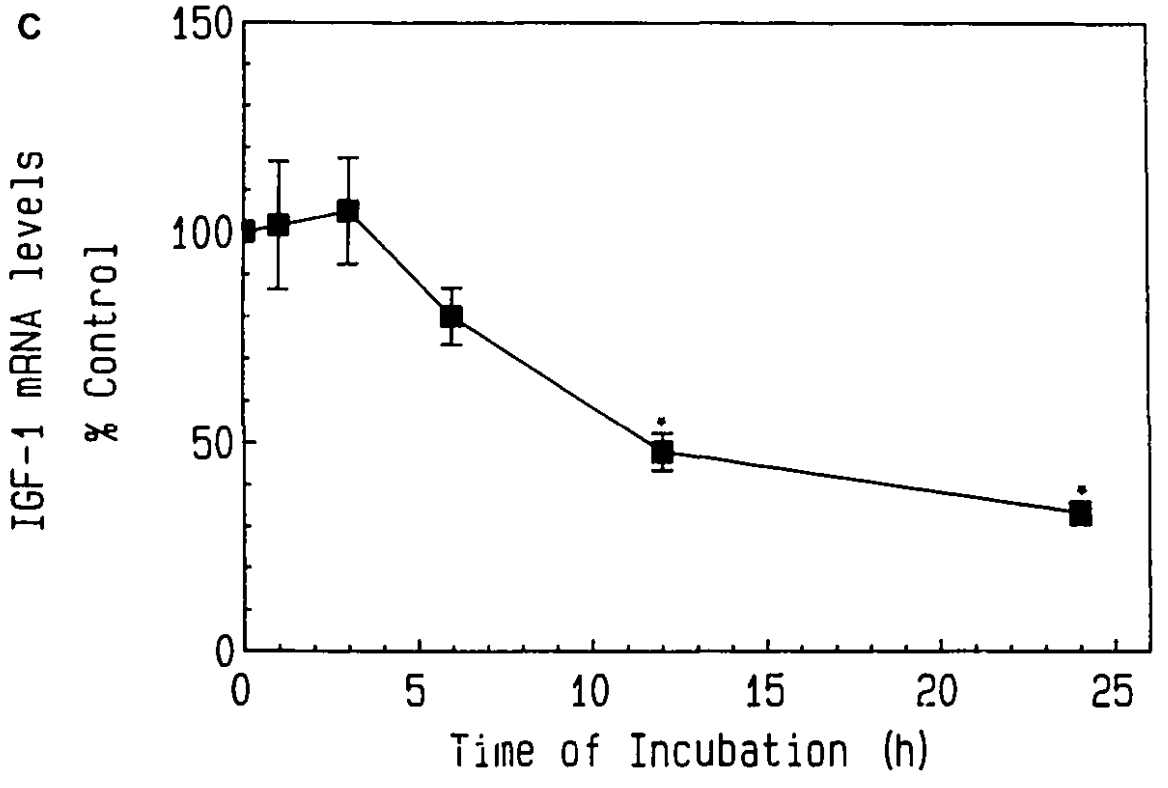


Figure 1

1 C. Quantitation of IGF-I mRNA. The normalized densitometric reading of the ratio of IGF-I mRNA to GAPDH mRNA are expressed as mean ± S.D. of triplicate determination by dot blot analyses. The values of IGF-I mRNA levels in all the samples are expressed as % of control which is normalized to 100%.

*p < 0.005 compared to control at 0 time.

Figure 1


C
IGF-1 mRNA levels
% Control
150
100
50
0
0
5
10
15
20
25
Time of Incubation (h)

three times in a serum free medium, the cells were incubated without or with bGH, glucagon and $(Bu)_2$cAMP and their combination in the presence of actinomycin D for 10 h. As shown in Table 2, IGF-I mRNA level in cells with no addition was 60% of the control at 0 time. bGH had no posttranscriptional effect on IGF-I mRNA stability, whereas glucagon and $(Bu)_2$cAMP in the presence or absence of bGH appear to stabilize IGF-I mRNA at a posttranscription level (75-90 % of control at 0 time).

Inhibition of protein stynthesis

Hepatocytes were incubated with 10 ug/ml cycloheximide for 6 h to inhibit protein synthesis. As shown in Table 3, protein synthesis measured by ^{3}H-leucine incorporation into proteins was inhibited 90% by cycloheximide.

bGH plus glucagon or bGH plus $(Bu)_2$cAMP stimulation of IGF-I mRNA levels require synthesis of a new protein(s)

We previously showed that bGH appeared to stimulate IGF-I mRNA levels by elevating PKC levels whereas glucagon's effect was via activation of PKA (manuscript in preparation). But PMA when added in combination with glucagon or $(Bu)_2$cAMP produced no synergistic effect on IGF-I mRNA stimulation indicating that activation or synthesis of one or more factor(s) or protein(s) may be required besides elevation of PKA or PKC.

The effect of GH on stimulation of c-fos, c-jun and c-myc mRNA (16,17) and the effect of cAMP on c-fos and c-myc mRNA

Table 2. Posttranscriptional effect of glucagon and $(Bu)_2$cAMP in the presence or absence of bGH on IGF-I mRNA levels in hepatocytes.

Treatment	IGF-I mRNA level % Control(at 0 time)
Control	59.23 ± 10.4
bGH	62.80 ± 12.5
Glucagon	84.91[a] ± 11.8
bGH + Glucagon	86.42[b] ± 7.6
$(Bu)_2$cAMP	89.08[c] ± 8.6
bGH + $(Bu)_2$cAMP	91.64[a] ± 11.8

[a]$p < 0.05$, [b]p 0.005, [c]$p < 0.01$ compared to control

72 h after plating, IGF-I mRNA levels in hepatocytes were stimulated by incubating the cells with 50 ng/ml bGH plus 100 ng/ml glucagon for 4 h. The hormones were removed by rinsing the cells in a serum free medium three times. The cells were incubated with fresh medium containing 5 ug/ml actinomycin D 30 min prior to adding various hormones or reagents. Total RNA was extracted at 0 time and rest of the cells were treated with no additions, 50 ng/ml bGH, 100 ng/ml glucagon, 50 ng/ml bGH plus 100 ng/ml glucagon, 100 uM $(Bu)_2$cAMP and 50 ng/ml bGH plus 100 uM $(Bu)_2$cAMP for 10 h in the presence of actinomycin D. At the end of incubation period, total RNA was extracted and subjected to Northern and dot blot analyses using IGF-I cDNA probe. The membranes were stripped and rehybridized with GAPDH cDNA probe. Normalized densitometric readings of the ratio of IGF-I mRNA to GAPDH mRNA are expressed as mean ± S.D. of triplicate determinations by dot blot analyses. The values of IGF-I mRNA levels in all the samples are expressed as % of control at 0 time, which is normalized to 100%.

Table 3. Effect of cycloheximide on ^{3}H-leucine incorporation into proteins in hepatocytes.

Treatment	^{3}H-leucine incorporation into proteins cpm	% protein synthesis	% inhibition of protein synthesis
Control	4889.30 ± 138.98	100	0
Cycloheximide	491.25 ± 39.16	10.05	89.9

Hepatocytes were cultured as described in Table 2. The cells were treated with 10 ug/ml of cycloheximide for 6 h. The cells were pulsed with 1 uci/ml ^{3}H-leucine 1 h before terminating the experiment. The proteins and nucleic acids were precipitated as described in Table 2 and ^{3}H-leucine incorporation into proteins was determined.

(18) suggest a requirement of protein synthesis on stimulation of IGF-I mRNA levels in rat liver. To investigate if bGH plus glucagon or bGH plus $(Bu)_2$cAMP require a new protein synthesis to produce a synergistic effect on IGF-I mRNA levels, the hepatocytes were incubated with cycloheximide 30 min prior to adding bGH, glucagon, $(Bu)_2$cAMP and their combinations. As shown in Figure 2, bGH effect on IGF-I mRNA stimulation does not appear to require a new protein synthesis whereas glucagon and $(Bu)_2$cAMP effect on IGF-I mRNA stimulation were inhibited 50% and 30% respectively in the presence of cycloheximide. Synergistic effect on IGF-I mRNA stimulation by bGH plus glucagon and bGH plus $(BU)_2$cAMP were inhibited 80% and 65% respectively in the presence of cycloheximide. These data indicate that bGH plus glucagon or bGH plus $(Bu)_2$cAMP require a synthesis of one or more proitein(s) to produce a synergistic effect on IGF-I mRNA levels. The effect of glucagon or $(Bu)_2$cAMP on IGF-I mRNA stimulation also appears to require synthesis of one or more new protein(s).

Figure 2. Effect of cycloheximide on bGH plus glucagon or bGH plus $(Bu)_2$cAMP stimulated IGF-I mRNA levels in hepatocytes.

2 A. Northern blot of IGF-I mRNA levels in hepatocytes. 72 h after plating, the cells were treated with 10 ug/ml of cycloheximide 30 min prior to adding various hormones or reagents. Hepatocytes were treated with no additions (lanes 1), with 50 ng/ml bGH (lanes 2), 100 ng/ml glucagon (lanes 3), 50 ng/ml bGH plus 100 ng/ml glucagon (lanes 4), 100 uM $(Bu)_2$cAMP (lanes 5) and 50 ng/ml bGH plus 100 uM $(Bu)_2$cAMP (lanes 6) for 6 h. cycloheximide was present throughout the incubation period. Total RNA samples prepared from hepatocytes were subjected to Northern blot and dot blot analyses using IGF-I cDNA probe.

2 B. Northern blot 2A was stripped and rehybridized with GAPDH cDNA probe to correct for the variation in total RNA applied.

Figure 2

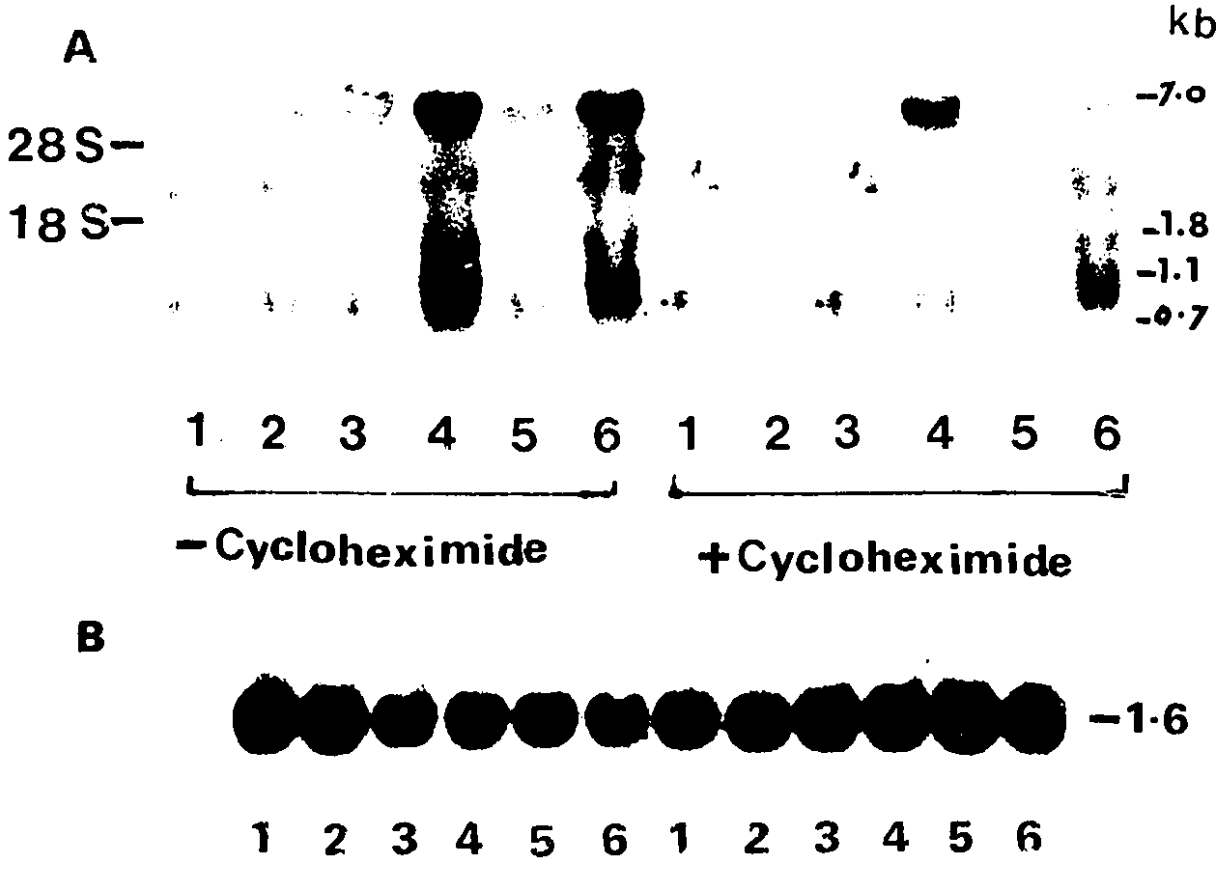

A
kb
28S–
18S–
7·0
1·8
1·1
0·7
1 2 3 4 5 6 1 2 3 4 5 6
–Cycloheximide
+Cycloheximide
B
1·6
1 2 3 4 5 6 1 2 3 4 5 6

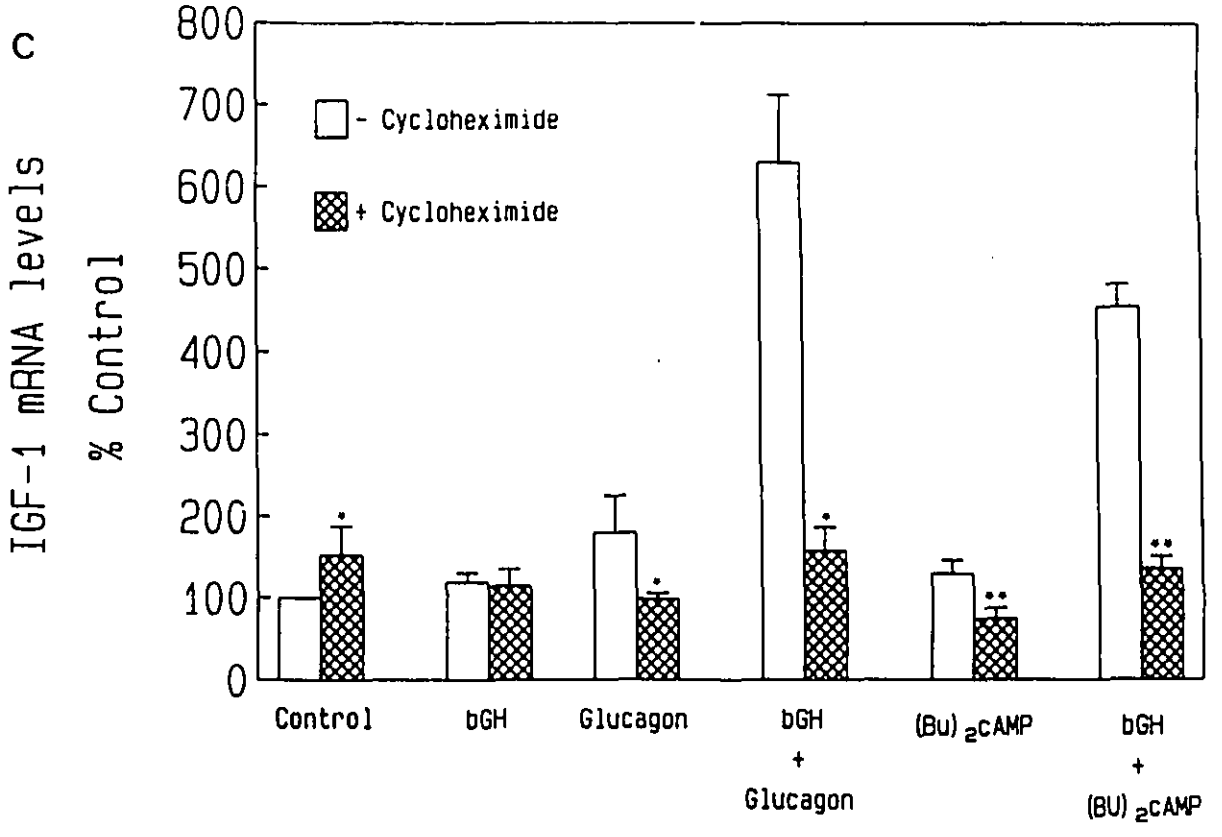
Figure 2

2 C. Quantitation of IGF-I mRNA. The normalized densitometric reading of the ratio of IGF-I mRNA to GAPDH mRNA is expressed as mean ± S.D. of triplicate determinations of total RNA by dot blot analyses. The values of all the samples are expressed relative to control which is normalized to 100%.

This experiment is representative of the two experiments carried out on various occasions and identical results were obtained.

$*p < 0.05$, $**p < 0.005$ compared to appropriate controls.

Figure 2


C
IGF-1 mRNA levels
% Control
800
700
600
500
400
300
200
100
0
- Cycloheximide
+ Cycloheximide
Control
bGH
Glucagon
bGH + Glucagon
(Bu) 2cAMP
bGH + (BU) 2cAMP

DISCUSSION

The rat IGF-I gene consists of 6 exons (19,20) and the sequence encoding the mature circulating 70 amino acid peptide resides within exons 3 and 4. Multiple IGF-I mRNA transcripts are generated by alternate splicing of exons 5 and 6 (21), use of different polyadenylation sites (22), use of multiple transcription initiation sites (23) and different promoter usage (24). The alternate splicing of exons 5 and 6 generate IGF-Ia (1.1 kb) and IGF-1b (1.8 kb) transcripts (21). The 7.6 kb mRNA was found to contain all the sequences present in the 1.1 kb IGF-Ia mRNA and in addition an unusually long 3' terminal untranslated sequence of over 6 kb. Two alternately used poly (A) addition sites within exon 6 derived part of IGF-I gene transcripts give rise to 1.1 kb and 7.6 kb mRNA species (25). The presence of AUUU sequence in 7.6 kb mRNA is known to destabilize a number of mRNAs (26).

In the present study, we have shown that the half-life of IGF-I mRNA in cultured rat hepatocyte is 12 h (Figure 1). In vivo in hypophysectomized rat, 7.0 kb hepatic IGF-I mRNA transcript has been shown to be less stable than the 0.7-1.1 kb transcript with their half-lives of 4 h and 14 h respectively (27). Our results are in agreement with those reported by Lowe WL jr et al (28) who showed that the half-lives of 7.0 kb and 0.8-1.2 kb IGF-I transcripts in GH_3 rat pituitary cell line were similar (10-12 h). The discrepancy between in vivo and in

vitro studies may be due to the use of actinomycin D in vitro, which has been shown to stabilize some short lived mRNAs apparently by neutralization of an AU rich destabilizing element in 3' UT region (29). Since 3' UT region of 7.6 kb mRNA is extremely rich in destabilizing AU sequence, actinomycin D may be stabilizing this transcript by neutralizing AU sequences.

Here we show that GH acts mainly at a transcriptional level to stimulate IGF-I mRNA accumulation and our results are in agreement with previous in vivo (4) and in vitro studies (30,31). Glucagon and $(Bu)_2$cAMP in the presence or absence bGH appear to act in part at a posttranscriptional level to stabilize IGF-I mRNA.

Agents that elevate intracellular levels of cAMP have been shown to mimick glucagon's effect on IGF-I mRNA accumulation when added in combination with bGH (manuscript in preparation). This indicates that glucagon may be mediating its effect on IGF-I mRNA accumulation via cAMP pathway. cAMP has been well documented in stabilization of a number of mRNAs. cAMP stabilizes phosphoenolpyruvate carboxykinase mRNA (32), osteocalcin mRNA (33) and chorionic gonadotropin mRNA (34). The mechanisms of mRNA stability by cAMP are not known, although mRNA stability is determined by specific sequences and/or structres in the transcripts (35,36). An increase in length of 3' poly (A) tail has been correlated with an increase in the stability of mRNA and the decrease in poly (A) tail appears to

destabilize mRNA (37). Poly (A) binding protein complexed to poly (A) tail also stabilizes mRNA (38) and the presence ofdestabilization sequence $(AUUU)_n$ in 3'-untranslated region makes mRNA unstable (26).

The early effect of GH to augment mRNA levels for c-fos, c-jun and c-myc (16,17) and that of cAMP to stimulate mRNA for c-fos and c-myc (18) suggest a requirement for protein synthesis in the augmentation of IGF-I mRNA levels in rat liver. We explored the relation between new protein synthesis and a synergistic effect on IGF-I mRNA stimulation produced by bGH plus glucagon or bGH plus $(Bu)_2$cAMP.

10 ug/ml cycloheximide which inhibited protein synthesis by 90% had no effect on bGH stimulated IGF-I mRNA levels. The inhibition of IGF-I mRNA stimulation by cycloheximide of glucagon, $(Bu)_2$cAMP, bGH plus glucagon and bGH plus $(Bu)_2$cAMP was respectively 50%, 30%, 80% and 65%. These results are consistent with the view that these agents require synthesis of one or more new protein(s) to effect stimulation of IGF-I mRNA levels in hepatocytes. Phosphorylated jun/fos heterodimer stimulated by phorbol ester binds and stimulates transcription from AP-1 enhancer sequence (39).

Since our results indicate that IGF-I mRNA accumulation stimulated by cAMP is cycloheximide sensitive, and if the effect of cAMP is on gene transcription, there may be an AP-2 binding site (40) present on IGF-I gene promoter. There is no evidence for the presence of AP-1 or AP-2 sites on IGF-1 gene promoter (11). These sites may be present elsewhere on IGF-I

gene (11).

In conclusion, we have shown that in hepatocytes, glucagon and $(Bu)_2$cAMP act partly at a posttranscriptional level to stabilize IGF-I mRNA. The synergistic effect of bGH plus glucagon or bGH plus $(Bu)_2$cAMP on the stimulation of IGF-I mRNA levels requires synthesis of one or more new protein(s). This is the first demonstration of these effects on IGF-I mRNA in hepatocytes. Whether glucagon or $(Bu)_2$cAMP stimuate IGF-I gene transcription, need further investigation. Identification and characterization of the proteins involved in bGH plus glucagon or bGH plus $(Bu)_2$cAMP effect on IGF-I mRNA stimulation will add to our understanding of regulation of IGF-I mRNA levels in rat hepatocytes.

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CHAPTER 6

BOVINE GROWTH HORMONE INHIBITS WHEREAS GLUCAGON STIMULATES IGFBP-1 mRNA LEVELS IN RAT HEPATOCYTES

PREFACE TO CHAPTER 6

As shown before, bGH inhibited whereas glucagon stimulated IGFBP(s) secretion by heptocytes in their culture medium. The effects of bGH, glucagon and various other hormones were investigated on IGFBP-1 mRNA levels in hepatocytes. This chapter deals with the mechanisms underlying bGH and glucagon effects on IGFBP-1 mRNA levels in hepatocytes.

Glucagon appeared to stimulate IGFBP-1 mRNA levels via PKA pathway whereas bGH appeared to act via stimulation of PKC to inhibit IGFBP-1 mRNA levels. In the presence of cycloheximide, IGFBP-1 mRNA was superinduced by bGH indicating the presence of a repressor protein induced by bGH. T_3 and insulin were found to inhibit, whereas dexamethasone was found to stimulate IGFBP-1 mRNA levels in hepatocytes. Insulin was found to be the most powerful inhibitor whereas glucagon and dexamethasone were the most potent stimulators of IGFBP-1 mRNA levels. Glucagon and dexamethasone acted by different mechanisms to stimulate IGFBP-1 mRNA levels as they produced an additive effect on IGFBP-1 mRNA stimulation when combined. Insulin inhibited IGFBP-1 mRNA levels in the presence of glucagon or dexamethasone. The effect of bGH on IGFBP-1 mRNA level was at the level of transcription whereas glucagon's effect was at a posttranscriptional level to stabilize IGFBP-1 mRNA in hepatocytes.

ABSTRACT

Primary cultured rat hepatocytes were used under serum free conditions to study the regulation of IGFBP-1 gene expression. IGFBP-1 mRNA levels were stimulated 2 to 3 fold by 200 ng/ml glucagon and inhibited 50 to 70 % by 50 ng/ml bovine growth hormone. This stimulatory effect of glucagon and the inhibitory effect of bGH were dose dependent. T_3 (20 nM) and insulin (100 nM) were found to inhibit IGFBP-1 mRNA levels 48 % and 95 % respectively, whereas dexamethasone (100 nM) and glucagon each were found to stimulate IGFBP-1 mRNA levels 3 to 4 fold. Dexamethasone and glucagon, when added together produced an additive effect and stimulated IGFBP-1 mRNA levels 7 to 8 fold. To probe the mechanism of glucagon's and bGH effect on IGFBP-1 mRNA levels, 3-isobutyl-1-methyl xanthine (IBMX), $(Bu)_2$cAMP and PMA were investigated. IBMX and $(Bu)_2$cAMP were found to stimulate, whereas PMA was found to inhibit IGFBP-1 mRNA levels in a dose dependent mannner. IGFBP-1 mRNA levels were stimulated 6 to 8 fold by 300 uM IBMX and 6 to 7 fold by 150 uM $(Bu)_2$cAMP, whereas 300 nM PMA inhibited IGFBP-1 mRNA levels 40 to 50 %. The inhibitory effect of bGH on IGFBP-1 mRNA levels were abolished in protein kinase C depleted cells and this results were confirmed by the use of inhibitors of protein kinases, staurosporine and H7. Glucagon's stimulatory effect on IGFBP-1 mRNA levels were blocked by the above inhibitors as they tend to inhibit protein kinase A as well as protein kinase C. bGH effect on IGFBP-1 mRNA levels appeared to require

synthesis of one or more protein(s). In the presence of 10 ug/ml cycloheximide IGFBP-1 gene is superinduced by bGH. The stimulatory effect of glucagon or $(Bu)_2$cAMP on IGFBP-1 mRNA does not appear to require synthesis of a new protein. Hepatic IGFBP-1 mRNA is short lived and has a half life of 2 h. bGH has no posttranscriptional effect on IGFBP-1 mRNA stability whereas glucagon and $(Bu)_2$cAMP stabilize IGFBP-1 mRNA at a posttranscriptional level.

In summary it appears that hepatic IGFBP-1 mRNA levels are inhibited by bGH, T_3 and insulin and stimulated by dexamethasone and glucagon. bGH may be mediating its effect on IGFBP-1 mRNA by activation of protein kinase C and it may require synthesis of one or more protein(s). In the presence of cycloheximide, bGH superinduces IGFBP-1 gene. Glucagon and $(Bu)_2$cAMP act in part at a posttranscriptional level to stabilize IGFBP-1 mRNA.

INTRODUCTION

Insulin-like growth factor (IGF) -I and -II do not exist as free peptides but are complexed to specific binding proteins (1). Various binding proteins with high affinity for IGFs have been detected in plasma and other biological fluids (2-6), tissue extract (7) and in the conditioned media from various cells in culture (8-12). The cDNAs for six rat IGF binding proteins (IGFBPs) have been cloned with numerical designations of IGFBP-1 (13), IGFBP-2 (14,15), IGFBP-3 (16,17), IGFBP-4 (18), IGFBP-5 (19) and IGFBP-6 (20). The physiological roles of these binding proteins are unknown. They may act synergistically with IGFs (21-24) or may inhibit (21-23) their action.

IGFBP-1 of molecular mass 30-32 kDa is present in high concentration in amniotic fluid (25) and fetal serum (26). The circulating levels of IGFBP-1 are increased in diabetes (27) and fasting (28,29) and decreases after insulin treatment and feeding respectively. IGFBP-1 mRNA is expressed in a number of fetal (30) and adult rat tissues (13). The expression of IGFBP-1 is high in fetal tissues and declines to a low level after birth, suggesting that this binding protein may have specific function during fetal develoment (30). IGFBP-1 gene is strongly induced in diabetes (30), fasting ((13,31) and hypophysectomy (32). IGFBP-1 mRNA is also expressed in various cell types in

culture (33-35).

Previous results from our laboratory showed that adult rat hepatocytes secrete a considerable amount of IGFBPs in culture medium. These BPs are stimulated 1.8-2.0 fold by 300 ng/ml glucagon and inhibited 82-85 % by 200 ng/ml bovine GH. From Western ligand blotting, 30-34 kDa IGFBPs secreted appeared to be IGFBP-1 and/or IGFBP-2, whereas 24 kDa IGFBP may be IGFBP-4 (10).

GH has been shown to mediate some of its effects via elevating DAG levels and activating protein kinase C (PKC) (36-38), whereas glucagon stimulates the production of inositol phosphate as well as that of cAMP in rat hepatocytes (39). Whether the inhibitory efffect of bGH and the stimulatory effect of glucagon on IGFBPs production observed previously (10) are at the level of mRNA or at the level of protein are not known.

This study was undertaken to demonstrate the effect of various hormones and their combinations on IGFBP-1 gene expression and to characterize bGH, glucagon and $(BU)_2$cAMP effect on IGFBP-1 mRNA levels. The relative contributions of protein kinase C (PKC), protein kinase A (PKA) and synthesis of new protein(s) on IGFBP-1 mRNA expression in cultured rat hepatocytes were also assessed.

Materials and Methods

Hormones and Chemicals

Bovine growth hormone (USDA-bGH-B-1, 1.4 IU/mg) was kindly supplied bu USDA Animal Hormone Program (Beltsville, MD). 4ß-phorbol 12ß-myristate 13α-acetate (PMA), 3-isobutyl-1-methyl xanthine (IBMX), N^6,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt, staurosporine, 1-(5-isoquinolinyl sulphonyl)-2-methyl piperazine (H7) free base, cycloheximide actinomycin D, glucagon, 3,5,3'-triiodothyronine (T_3), dexamethasone and insulin were purchased from Sigma chemical Co. (St. Louis, Mo). The chemicals and reagents used for liver perfusion, hepatocyte cultures, RNA extraction and Northern blot analyses were obtained as indicated previously (10).

Hepatocyte cultures and hormonal stimulation

Hepatocytes were isolated from male Sprague Dawley rats by perfusion <u>in situ</u> with collagenase (40,41) and maintained in a serum free medium as described previously (10). Unless otherwise stated, the hepatocytes were stimulated with various hormones, reagents or their combinations for 6 h. after 72 h of plating.

RNA extraction, Northern and dot blot analyses

At the end of incubation period, total RNA was extracted from the hepatocytes by guanidinium isothiocyanate cesium chloride teqhnique (42,43) with slight modifications (10). 40

ug of total RNA was size fractionated on 1.5 % agarose, 2.2 M formaldehyde denaturing gel and transfered to a hybond-N-nylon membrane by capillary transfer as described previously (10). Dot blot analyses was carried out on 5 ug of total RNA in triplicate in a dot blot manifold from Bio-Rad using the manufacturer's protocol. RNA was fixed to the membranes by heating in an oven at 80 C for 2 h and hybridized with 0.5 x 10^6 cpm/ml of ^{32}P-labeled IGFBP-1 cDNA probe (1 kb) (13). The membranes were stripped using the manufacturer's protocol for hybond-N-membranes and rehybridized with 0.5 x 10^6 ^{32}P-labeled GAPDH cDNA probe (750 bp) to normalize for possible variations in gel loading and transfer of RNA. The cDNA probes were labeled to a specific activity of 10^9 dpm/ug DNA using random primer DNA labeling system. The hybridization and washing conditions for the membranes were as described before (10).

Densitometric quantitation

RNA samples in each experiment were analysed by Northern blot as well as by dot blot analyses in triplicate. IGFBP-1 mRNA levels in the Northern and dot blots were quantitated by scaning in an LKB Ultrascan XL enhanced laser densitometer. The results are expressed as mean ± S.D. of triplicate determinations of the ratio of IGFBP-1 mRNA to GAPDH mRNA levels of dot blots. The levels of IGFBP-1 mRNA in all the samples are expressed relative to control which is normalized to 100 %.

Statistical analyses

Statistical analyses were carried out by paired t-test. A value of $p < 0.05$ was considered significant.

RESULTS

Time dependent effect of bGH and/or glucagon on IGFBP-1 mRNA levels.

We investigated if IGFBP-1 mRNA was expressed in cultured rat hepatocytes and if the stimulatory effect of glucagon and the inhibitory effect of bGH observed on IGFBPs production (10) are at the level of mRNA. A time dependent study of bGH and/or glucagon effect on IGFBP-1 mRNA level was carried out as shown in Figure 1. Glucagon stimulated IGFBP-1 mRNA levels 3 fold after 1 h of incubation, reached a maximum stimulation after 3 h and gradually declined to a baseline level. bGH inhibited IGFBP-1 mRNA levels after 3 h of incubation and this effect was persistant till 12 h and reached a baseline level by 24 h of incubation. When glucagon was added in combination with bGH, the stimulatory effect on IGFBP-1 mRNA level was slightly less than that observed with glucagon alone, indicating that bGH and glucagon act via different mechanisms to mediate their effects on IGFBP-1 mRNA levels.

Dose dependent effect of bGH or glucagon on IGFBP-1 mRNA levels

Northern blot of total RNA samples from hepatocytes was performed using IGFBP-1 cDNA probe as described in Materials and Methods. bGH shows a dose dependent inhibition (Fig. 2A, upper panel), whereas glucagon shows a dose dependent stimulation (Fig. 2B, upper panel) of IGFBP-1 mRNA levels. The membranes were stripped and subsequentlty rehybridized with

Figure 1. Time dependent effect of bGH and/or glucagon on IGFBP-1 mRNA levels in hepatocytes.

Hepatocytes were prepared and cultured as described previously (10). 72 h after plating, the cells were treated with 200 ng/ml bGH, 300 ng/ml glucagon and 200 ng/ml bGH plus 300 ng/ml glucagon for various time periods as shown in the figure. Total RNA was prepared from various samples after hormonal treatment and subjected to Northern blot analyses using IGFBP-1 cDNA probe as described in Materials and Methods. The membrane was stripped and subsequently reprobed with GAPDH cDNA probe to normalize for possible variations in gel loading and transfer of RNA. IGFBP-1 and GAPDH mRNAs were quantitated by densitometric scanning. Normalized IGFBP-1 mRNA levels are expressed as the ratio of IGFBP-1 mRNA to GAPDH mRNA. All the values are expressed as % control which are normalized to 100 % at each time point.

This experiment is representative of the two experiments carried out on different occasions and identical results were obtained.

Figure 1

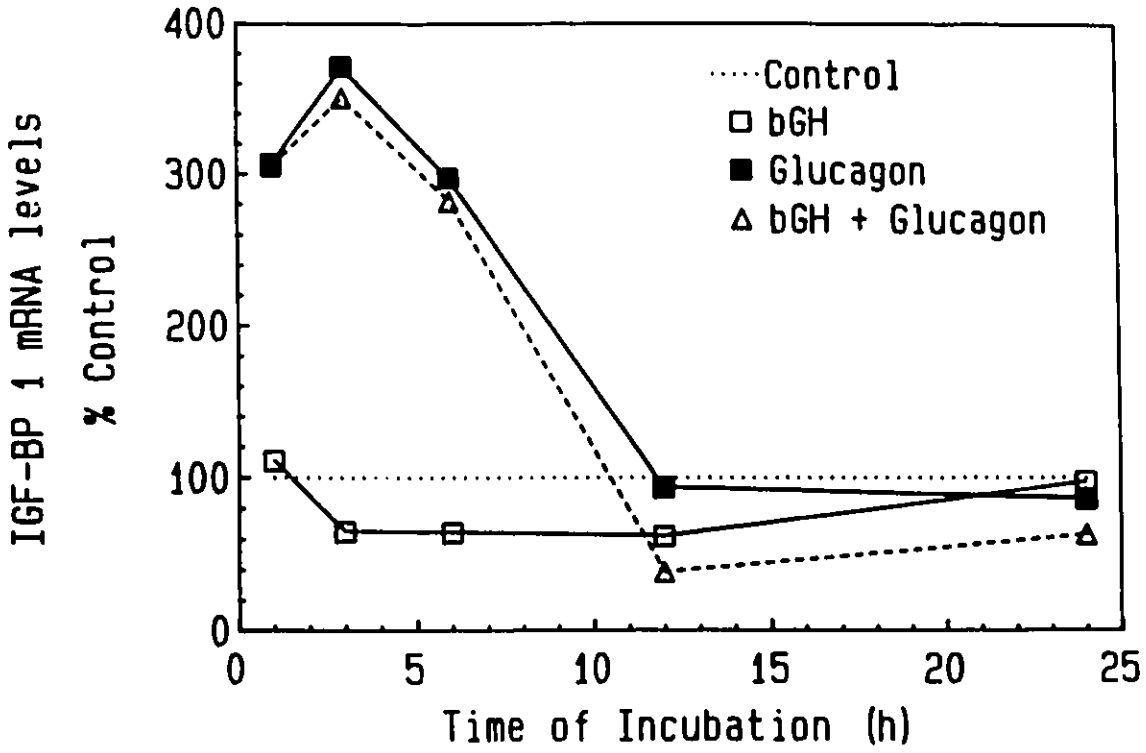

IGF-BP 1 mRNA levels
% Control
400
300
200
100
0
0
5
10
15
20
25
Time of Incubation (h)
Control
bGH
Glucagon
bGH + Glucagon

Figure 2. Dose dependent effect of bGH and glucagon on IGFBP-1 mRNA levels.

Fig. 2A and 2B. Northern blots of IGFBP-1 mRNA levels and GAPDH mRNA levels in hepatocytes. Hepatocytes were treated with various concentraions of bGH or glucagon for 6 h. Total RNA prepared from the samples were subjected to Northern blot analyses using IGFBP-1 cDNA probe (upper panel). The membranes were stripped and subsequently rehybridized with GAPDH cDNA probe (lower panel).

Figure 2

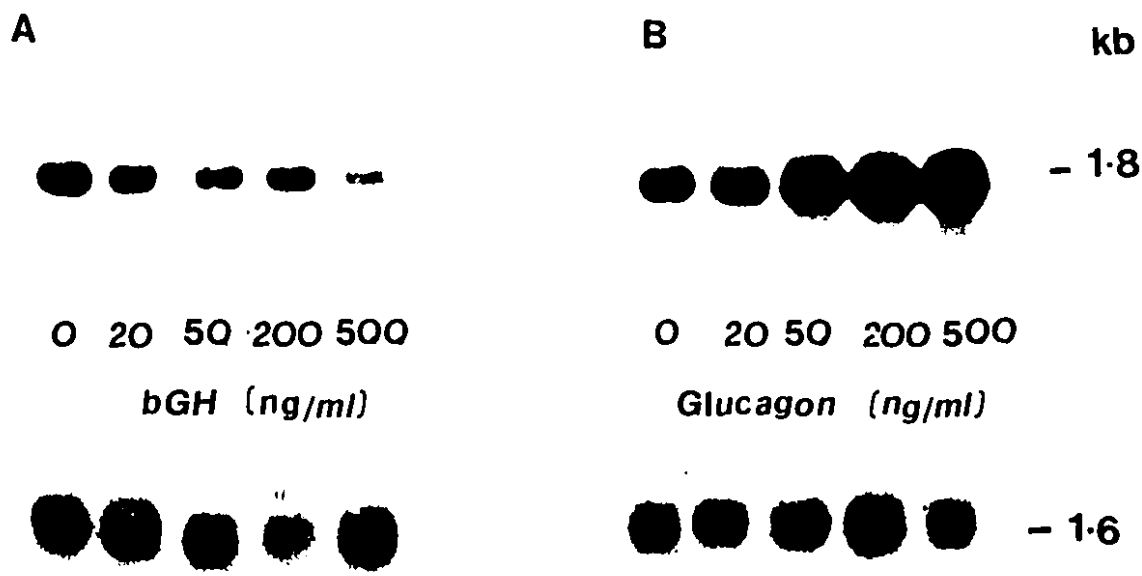
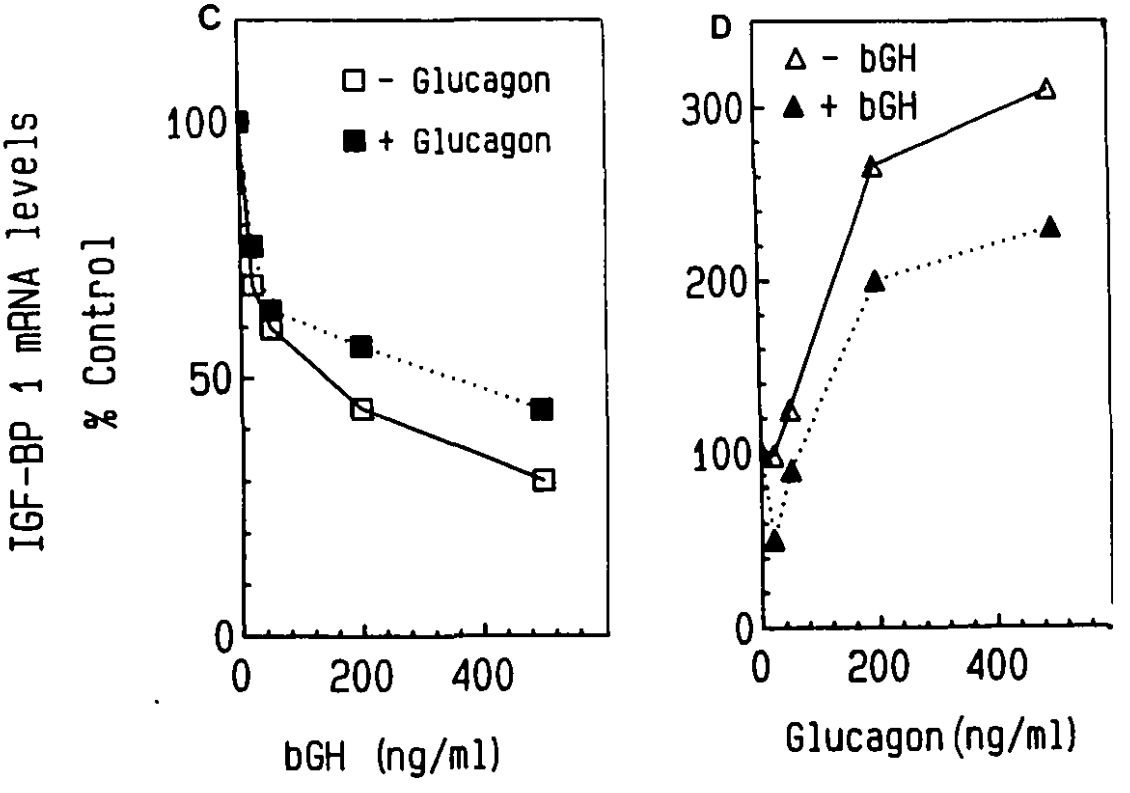


Figure 2

Fig. 2C and 2D. Quantitation of IGFBP-1 mRNA levels in Northern blots 2A and 2B. IGFBP-1 mRNA levels were quantitated and normalized as described in Fig. 1. Dose dependent inhibitory effect of bGH on IGFBP-1 mRNA levels in the presence and absence of 100 ng/ml glucagon is shown in Figure 2C. Dose dependent stimulatory effect of glucagon in the presence and absence of 50 ng/ml bGH is shown in Figure 2D. Northern blots of dose dependent inhibitory effect of bGH in the presence of glucagon and dose dependent stimulatory effect of glucagon in the presence of bGH are not shown.

Figure 2


C
□ - Glucagon
■ + Glucagon
100
50
0
0
200
400
bGH (ng/ml)
IGF-BP 1 mRNA levels
% Control
D
△ - bGH
▲ + bGH
300
200
100
0
0
200
400
Glucagon (ng/ml)

GAPDH cDNA probe (Fig. 2A and 2B lower panel) to normalize for the possible variations in gel loading and transfer of RNA. IGFBP-1 mRNA levels in Figure 2A and 2B are quantitated, normalized and expressed as % control as described in Fig. 1. As shown in Figure 2C, bGH shows a dose dependent inhibition of IGFBP-1 mRNA levels in the presence or absence of 100 ng/ml glucagon. 500 ng/ml bGH inhibits IGFBP-1 mRNA levels 70 % of the control in the absence of glucagon, whereas it inhibits IGFBP-1 mRNA levels 50 % of the control in the presence of glucagon. Glucagon shows a dose dependent stimulation of IGFBP-1 mRNA levels in the presence or absence of 50 ng/ml bGH as shown in Figure 2D. 500 ng/ml glucagon shows 3 fold stimulation of IGFBP-1 mRNA levels in the absence of bGH, whereas it shows 2 fold stimulation in the presence of bGH.

Effect of various hormones and their combinations on IGFBP-1 mRNA levels

Dexamethasone has been shown to stimulate (44), whereas insulin has been shown to inhibit (35) IGFBP-1 gene expression in H4-II-E rat hepatoma cells. Effect of T_3, insulin, dexamethasone and their combinations in the presence and absence of bGH or glucagon on IGFBP-1 mRNA levels were investigated. As indicated in Table 1, bGH, T_3 and insulin inhibited, whereas glucagon and dexamethasone stimulated IGFBP-1 mRNA levels. Insulin is the most potent inhibitor and inhibited IGFBP-1 mRNA levels 95 % of the control. It also

Table 1. Effect of various hormones and their combinations on IGF-BP 1 mRNA levels in hepatocytes.

Treatment	IGF-BP 1 mRNA levels % Control	
bGH	34.34^{a} ±	1.7 *
T_3	52.88^{a} ±	2.3 *
Ins.	4.33^{b} ±	1.5 *
Glucagon	447.68^{a} ±	46.6 *
Dex.	371.41^{a} ±	28.5 *
Dex. + T_3	326.50 ±	56.1
+ bGH	185.18^{b} ±	20.7 ▲
+ Ins.	27.95^{a} ±	3.6 ▲
+ Glucagon	729.08^{a} ±	66.2 ▲
Glucagon + bGH	298.51^{c} ±	25.6 ▼
+ Ins.	14.19^{a} ±	2.1 ▼

$^{a}p < 0.005$, $^{b}p < 0.001$, $^{c}p < 0.05$

* compared to control, ▲ compared to Dex. treated control, ▼ compred to glucagon treated control.

Hepatocytes were treated with 50 ng/ml bGH, 20 nM T_3, 100 nM insulin, 100 ng/ml glucagon and 100nM dexamethasone and their combinations for 6 h. Total RNA prepared from the samples were subjected to Northern and dot blot analyses using IGFBP-1 cDNA probe. The membranes were stipped and rehybridized with GAPDH cDNA probe. IGFBP-1 mRNA was quantitated and normalyzed as described in Fig.1. IGFBP-1 mRNA levels in all the samples are expressed as mean ± S.D. of triplicate samples analysed by dot blot anlyses and expressed as % control.

abolished the stimulatory effect of dexamethasone and glucagon on IGFBP-1 mRNA levels. T_3 is a weak inhibitor and inhibited IGFBP-1 mRNA levels 50 % of control. Unlike insulin, T_3 has a slight inhibitory effect on stimulation of IGFBP-1 mRNA levels by dexamethasone. bGH appears to be a more potent inhibitor than T_3 but not as powerful an inhibitor as insulin. Glucagon and dexamethasone both appear to be equally powerful stimulators of IGFBP-1 mRNA levels and show an additive effect on IGFBP-1 mRNA stimulation when combined. This indicates that glucagon and dexamethasone may be acting via different mechanisms to stimulate IGFBP-1 mRNA levels in hepatocytes.

Effect of IBMX, $(Bu)_2$cAMP and PMA on IGFBP-1 mRNA levels

Glucagon has been shown to act via stimulation of cAMP levels or by the production of inositol phosphate in rat hepatocytes (39). To assess the role of agents that elevate intracellular levels of cAMP and PKC on IGFBP-1 mRNA levels, the cells were treated with varying concentrations of IBMX (Fig. 3A), $(Bu)_2$cAMP (Fig. 3B) and PMA (Fig.3C). The upper panel shows the Northern blot of IGFBP-1 mRNA levels. The membranes were stripped and subsequently rehybridized with GAPDH cDNA probe to normalize for the possible variations in gel loading and transfer of RNA (Fig. 3A, 3B and 3C lower panel). IGFBP-1 mRNA levels were quantitated, normalized and expressed as % control as described in Fig. 1. As shown in Figure 3D, IGFBP-1 mRNA level is stimulated 6 to 8 fold by

Figure 3. Dose dependent effect of IBMX, $(Bu)_2$cAMP and PMA on IGFBP-1 mRNA levels in hepatocytes.

Fig. 3A, 3B and 3C. Northern blots of IGFBP-1 mRNA levels (upper panel) and GAPDH mRNA levels (lower panel). Hepatocytes were treated with various concentrations of IBMX, $(Bu)_2$cAMP and PMA for 6 h. Total RNA samples prepared were subjected to Northern blot analyses using IGFBP-1 cDNA probe as described in Fig. 1. The membranes were stripped and reprobed with GAPDH cDNA.

Figure 3

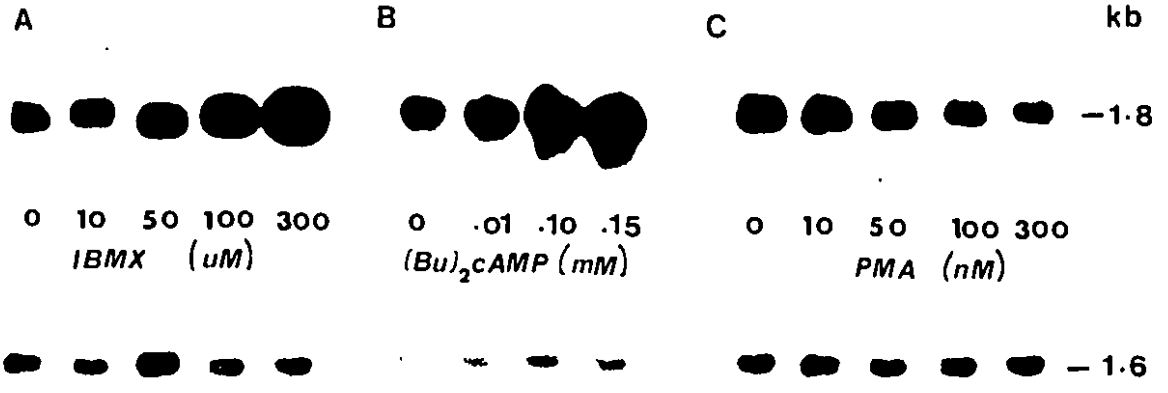
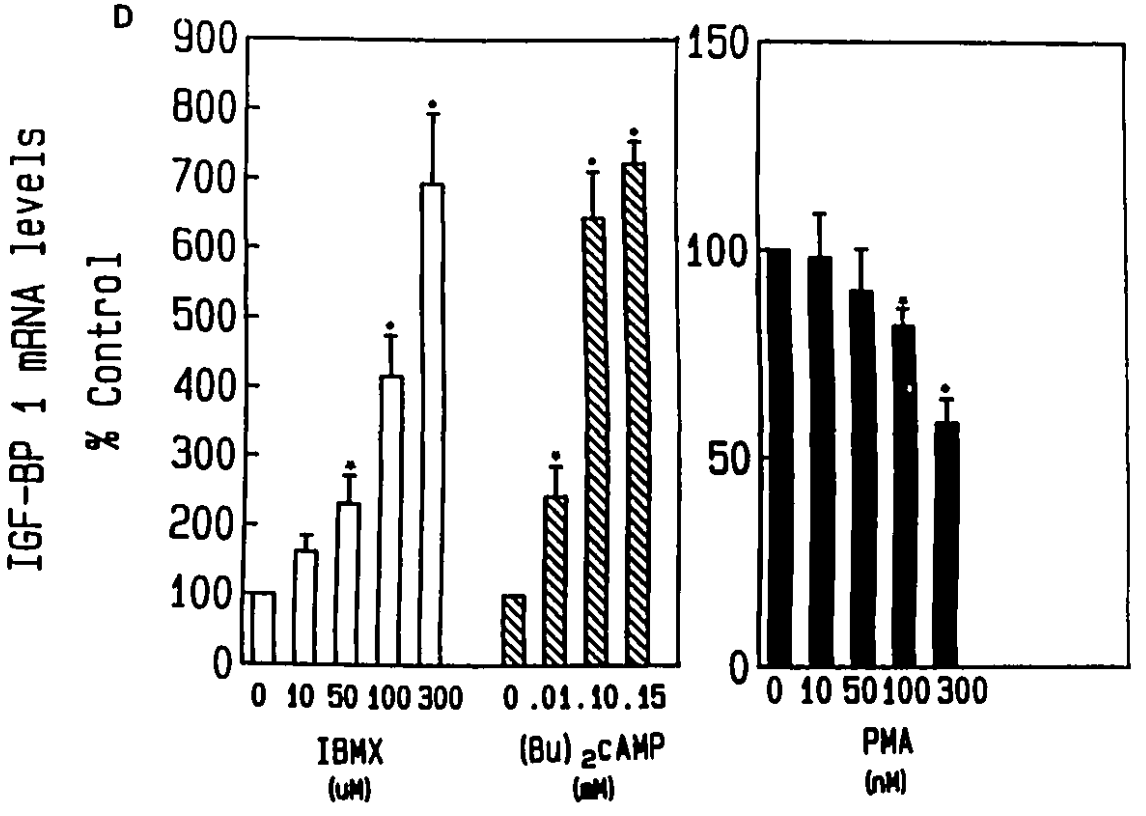

A
B
C
kb
1·8
1·6
0 10 50 100 300
IBMX (uM)
0 .01 .10 .15
(Bu)2cAMP (mM)
0 10 50 100 300
PMA (nM)

Figure 3

Fig. 3D. IGFBP-1 mRNA levels were quantitated and normalized as described in Fig. 1. Normalized IGFBP-1 mRNA levels are expressed as mean ± S.D. of triplicate determinations by dot blot anaylses

★ $p < 0.01$, ◆ $p < 0.005$

Figure 3


D
IGF-BP 1 mRNA levels
% Control
900
800
700
600
500
400
300
200
100
0
0 10 50 100 300
IBMX
(uM)
0 .01 .10 .15
(Bu) 2cAMP
(mM)
150
100
50
0
0 10 50 100 300
PMA
(nM)

300 uM IBMX and 6.5 to 7.5 fold by 150 uM $(Bu)_2$cAMP. 300 nM PMA inhibited IGFBP-1 mRNA levels 50 % of control.

Effect of bGH and glucagon on IGFBP-1 mRNA levels in PKC depleted hepatocytes

GH has been shown to mediate some of its efffects via activation of PKC (36-38). Our results in Fig. 3C and 3D, where PMA inhibited IGFBP-1 mRNA levels correlate closely to the effect of bGH observed on IGFBP-1 mRNA levels in Fig. 2A and 2C. To confirm our observations that bGH may be acting via PKC activation to produce an inhibitory effect on IGFBP-1 mRNA levels and that glucagon does not appear to act via PKC pathway to produce a stimulatory effect on IGFBP-1 mRNA levels, the effect of bGH, glucagon and bGH plus glucagon were investigated in PKC depleted hepatocytes. As shown in Table 2, bGH has no inhibitory effect on IGFBP-1 mRNA levels in PKC depleted cells. Glucagon is able to stimulate IGFBP-1 mRNA levels in PKC depleted cells to the same extent as in control cells. The inhibitory effect of bGH when added in combination with glucagon is not observed in PKC depleted cells when compared to control cells.

Effect of inhibitors of protein kinases on IGFBP-1 mRNA levels

To confirm whether protein kinases were involved in bGH and glucagon effect on IGFBP-1 mRNA levels, the effect of inhibitors of protein kinases were investigated. As shown in Figure 4, bGH inhibited IGFBP-1 mRNA levels 50 % of control and

Table 2 Effect of bGH and glucagon on IGF-BP 1 mRNA levels in PKC depleted hepatocytes.

Treatment	IGF-BP 1 mRNA levels in hepatocytes	
	preincubated with vehicle	preincubated with PMA
Control	100	103.88 ± 5.6
bGH	52.92 ± 8.2	129.28[a] ± 7.8
Glucagon	241.63 ± 12.9	266.53[b] ± 5.5
bGH + Glucagon	130.56 ± 18.3	288.15[c] ± 21.7

[a] $p < 0.005$, [b] $p < 0.05$, [c] $p < 0.0.001$

compared to approprate controls preincubated with vehicle.

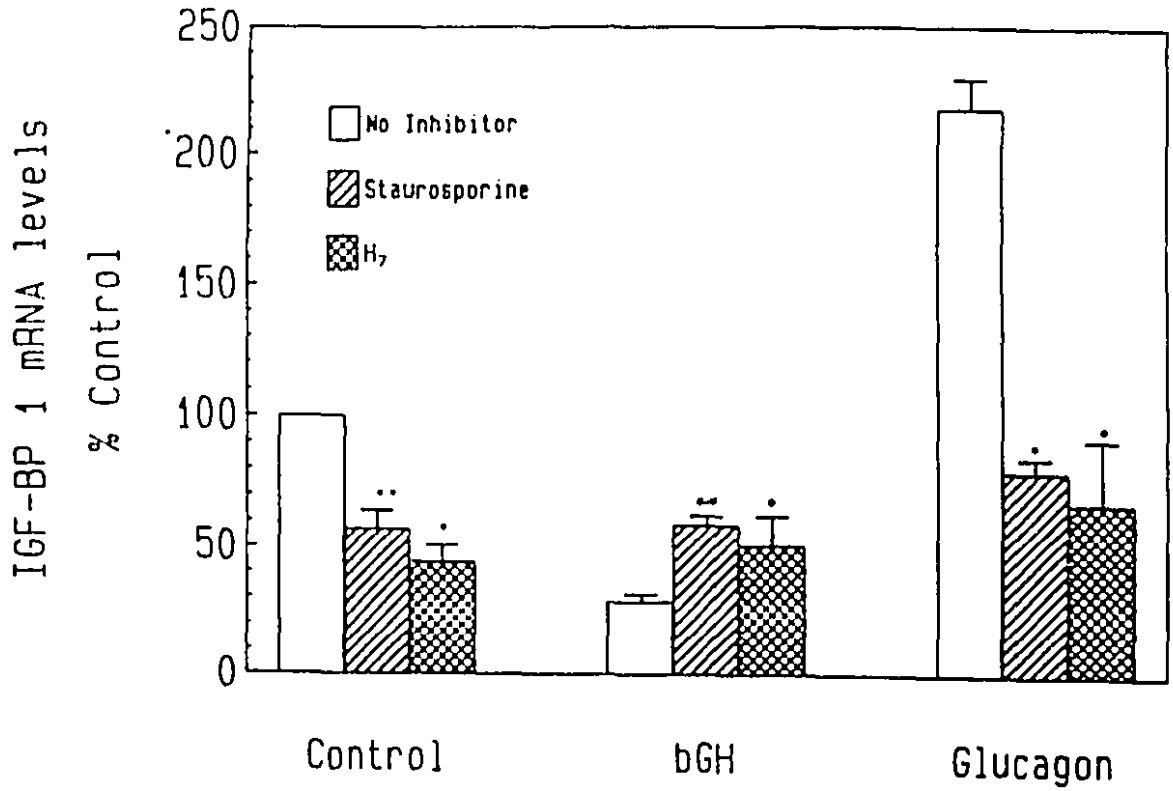
Hepatocytes were treated with vehicle or PMA for 24 h. The cells were rinsed 3 times with serum free medium and incubated with fresh medium containing 50 ng/ml bGH, 100 ng/ml glucagon and 50 ng/ bGH plus 100 ng/ml glucagon for 6 h. Total RNA samples prepared were subjected to Northern and dot blot anlyses using IGFBP-1 cDNA probe. The membranes were stripped and reprobed with GAPDH cDNA. IGFBP-1 mRNA was quantitated and normalized as described in Fig.1. IGFBP-1 mRNA levels are expressed as mean ± S.D. of triplicate determinations by dot blot anlyses.

Figure 4. Effect of inhibitors of protein kinases on bGH inhibited and glucagon stimulated IGFBP-1 mRNA levels.

Hepatocytes were treated with 200 nM staurosporine and 1 uM H7 30 min prior to the addition of 50 ng/ml bGH and 100 ng/ml glucagon for 6 h. Total RNA samples prepared were subjected to Northern and dot blot analyses using IGFBP-1 cDNA probe. The membranes were stripped and rehybridized with GAPDH cDNA probe. IGFBP-1 mRNA was quantitated and normalized as described in Fig. 1. IGFBP-1 mRNA level is expressed as mean ± S.D. of triplicate determination by dot blot analyses. IGFBP-1 mRNA levels in all the samples are expressed relative to control.

★ $p < 0.05$, ★★ $p < 0.01$, ♦ $p < 0.005$

Figure 4


IGF-BP 1 mRNA levels
% Control
250
200
150
100
50
0
No Inhibitor
Staurosporine
H7
Control
bGH
Glucagon

glucagon stimulated IGFBP-1 mRNA levels 1.8 to 2 fold. These effects of bGH and glucagon were abolished in the presence of staurosporine (45) and H7 (46) which have been shown to inhibit PKA as well as PKC. These results indicate that bGH may be acting via stimulating PKC levels to inhibit IGFBP-1 mRNA levels whereas glucagon may be acting via PKA pathway to stimulate IGFBP-1 mRNA levels.

Effect of cycloheximide on bGH inhibited or glucagon and $(Bu)_2$cAMP stimulated IGFBP-1 mRNA levels

A role of new protein synthesis for bGH, glucagon or $(Bu)_2$cAMP effect on IGFBP-1 mRNA levels was assessed. The cells were preincubated with 10 ug/ml cycloheximide 30 min prior to the addition of various hormones or reagents. This dose of cycloheximide was sufficient to inhibit protein synthesis by 92 %. As shown in Table 3, bGH appears to superinduce IGFBP-1 mRNA in the presence of cycloheximide. This property of bGH is also noticable when added in combination with glucagon or $(Bu)_2$cAMP. The stimulatory effect of glucagon or $(Bu)_2$cAMP on IGFBP-1 mRNA levels are not dependent on new protein synthesis.

Half life of IGFBP-1 mRNA

Figure 5 shows the decay rate of IGFBP-1 mRNA measured over a 24 h period in the presence of 5 ug/ml actinomycin D. This dose of actinomycin D was sufficent to inhibit RNA synthesis by 95 %. IGFBP-1 mRNA is short lived and has a half life of 2 h.

Table 3 Effect of bGH, glucagon and $(Bu)_2$cAMP in the presence and absence of cycloheximide on IGF-BP 1 mRNA levels

Treatment	Cycloheximide (10 ug/ml)	IGF-BP 1 mRNA levels % Control	
Control	-	100	
Control	+	125.97 ± 10.7	
bGH	-	50.61 ± 3.3	
bGH	+	289.41[a] ± 35.7	*
Glucagon	-	611.23 ± 91.3	
Glucagon	+	651.75 ± 69.5	
Glucagon + bGH	-	317.71 ± 14.2	
Glucagon + bGH	+	700.04[b] ± 63.7	▲
$(Bu)_2$cAMP	-	594.89 ± 92.9	
$(Bu)_2$cAMP	+	518.12 ± 22.6	
$(Bu)_2$cAMP + bGH	-	455.34 ± 29.0	
$(Bu)_2$cAMP + bGH	+	710.62[c] ± 90.8	▼

[a] $p < 0.005$, [b] $p < 0.01$, [c] p 0.05

* compared to bGH treated control, ▲ compared to glucagon + bGH treated control, ▼ compared to $(Bu)_2$cAMP + bGH treated control.

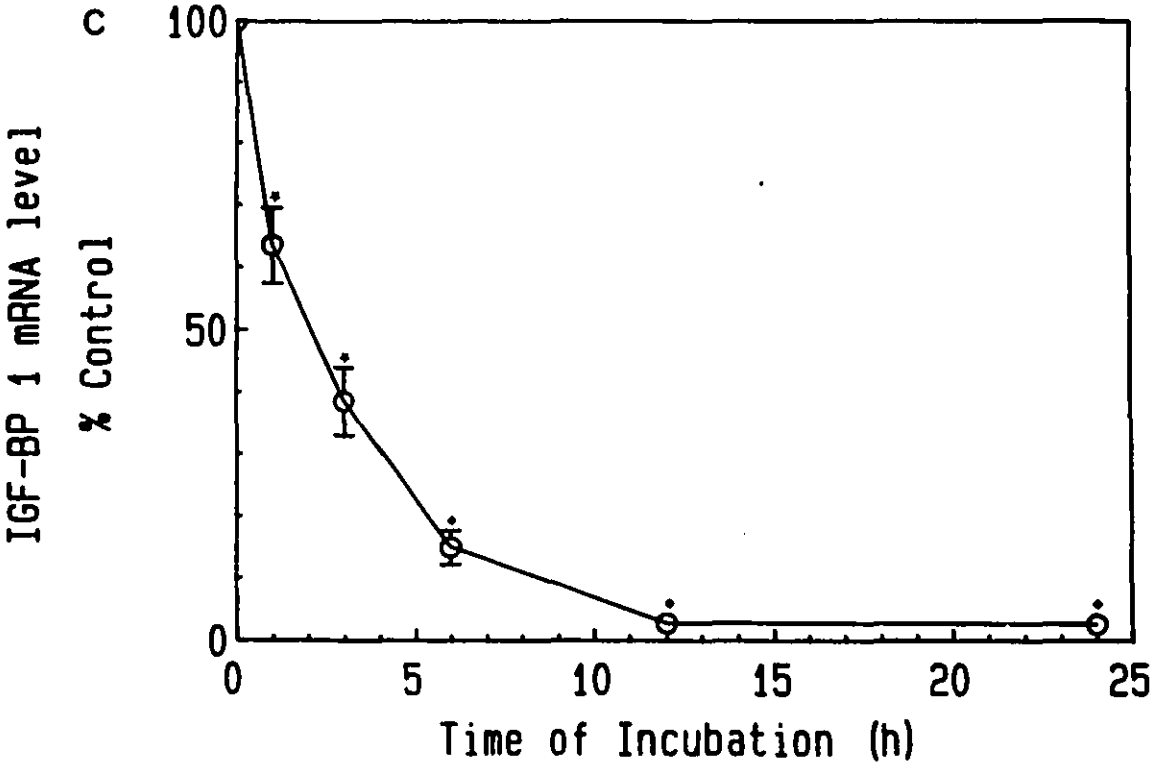
Hepatocytes were treated with 10 ug/ml cycloheximide 30 minutes prior to the addition of 50 ng/ml bGH, 100 ng/ml glucagon, 100 uM $(Bu)_2$cAMP and their combination for 6 h. Total RNA samples prepared were subjected to Northern and dot blot analyses using IGFBP-1 cDNA probe. The membranes were stripped and rehybridized with GAPDH cDNA probe. IGFBP-1 mRNA levels were quantitated and normalized as described in Fig.1. Normalized IGFBP-1 mRNA levels are expressed as mean ± S.D. of triplicate determinations by dot blot analyses.

Figure 5. Half life of IGFBP-1 mRNA in hepatocytes.

The decay rate of IGFBP-1 mRNA was measured at various time of incubation in the presence of 5 ug/ml actinomycin D. Hepatocytes were treated with 5 ug/ml actinomycin D and total RNA was extracted after various times of incubation. RNA samples were subjected to Northern and dot blot analyses using IGFBP-1 cDNA probe. The membranes were stripped and rehybridized with GAPDH cDNA probe. IGFBP-1 mRNA was quantitated and normalized as described in Fig.1. Normalized IGFBP-1 mRNA levels are expressed as mean ± S.D. of triplicate determinations by dot blot analyses.

★$p < 0.001$, ◆$p < 0.005$

Figure 5


C
100
50
0
IGF-BP 1 mRNA level
% Control
0
5
10
15
20
25
Time of Incubation (h)

Posttranscriptional effect of bGH, glucagon and $(Bu)_2$cAMP on IGFBP-1 mRNA levels

Decay of IGFBP-1 mRNA was measured after 4 h of incubation with bGH, glucagon and $(Bu)_2$cAMP in the presence of actinomycin D. The cells were treated with 5 ug/ml actinomycin D 30 min prior to the addition of various hormones or agents. As shown in Table 4, bGH appears to have no postranscriptional effect on IGFBP-1 mRNA stability, whereas glucagon and $(Bu)_2$cAMP appear to stabilize IGFBP-1 mRNA at a posttranscriptional level.

Table 4 Posttranscriptional effect of bGH, glucagon and $(Bu)_2$cAMP on IGF-BP 1 mRNA levels in hepatocytes.

Treatment in the presenence of Act. D	IGF-BP 1 mRNA levels % Control at 0 time
Control	38.22 ± 6.7
bGH	39.50 ± 10.1
Glucagon	58.88[a] ± 5.1
$(Bu)_2$cAMP	54.14[b] ± 4.1

[a] $p < 0.05$, [b] $p < 0.01$ compared to control after 6 h incubation in the presence of Act. D.

Hepatocytes were treated with 5 ug/ml actinomycin D 30 min prior to the addition of 50 ng/ml bGH, 100 ng/ml glucagon or 100 uM $(Bu)_2$cAMP for 4 h. Total RNA samples prepared from the hepatocytes were subjected to Northern and dot blot analysis using IGFBP-1 cDNA probe. The membranes were stripped and reprobed with GAPDH cDNA. IGFBP-1 mRNA levels were quantitated and normalized as described in Fig.1. IGFBP-1 mRNA levels are expressed as mean ± S.D. of triplicate determinations of dot blot analyses. IGFBP-1 mRNA levels in all the samples are expressed as % control at 0 time.

DISCUSSION

IGFBP-1 mRNA observed as a single transcript of 1.8 kb is expressed in vivo in adult (13) and fetal rat tissues (30). hepatic IGFBP-1 gene is strongly induced in diabetes (30) and hypophysectomy (32) and reaches control levels by insulin (30) and GH (32) treatment respectively. Whether this increase in hypophysectomy reflects insulin deficiency or GH deficiency (30,32) is presently unclear.

In the present study, we demonstrate the inhibitory effect of bGH on IGFBP-1 mRNA levels in cultured rat hepatocytes. A time dependent inhibitory effect of bGH on IGFBP-1 mRNA levels indicate that inhibition by bGH is evident between 3 to 12 h of incubation and reaches a baseline level by 24 h incubation. We also report the stimulatory effect of glucagon on IGFBP-1 mRNA levels in hepatocytes. This effect of glucagon is observed after 1 h of incubation, reaches apeak at 3 h and gradually declines to a baseline level by 12 h of incubation. The inhibitory effect of bGH and the stimulatory effect of glucagon are dose dependent. bGH was found to inhibit IGFBP-1 mRNA levels in the presence of glucagon when compared to glucagon treated control. This suggests that bGH and glucagon may be acting via different mechanisms to regulate IGFBP-1 mRNA levels in hepatocytes. Mechanisms of bGH and glucagon effect on IGFBP-1 mRNA levels were investigated. GH has been well documented in mediating some of its effects via elevating DAG levels (36,37)

and activating PKC (37,38). To investigate if the bGH effect on IGFBP-1 mRNA levels is mediated via PKC pathway, hepatocytes were treated with PMA. PMA was found to inhibit IGFBP-1 mRNA levels in a dose dependent manner and this inhibiton was in close approximation to that observed with bGH. bGH and glucagon effects on IGFBP-1 mRNA levels were also tested in PKC depleted cells. Prolonged treatment with PMA has been shown to reduce cellular levels of PKC (47). bGH was found to produce no inhibitory effect on IGFBP-1 mRNA levels by itself or when added in combination with glucagon in PKC depleted cells. These results were further confirmed by the use of inhibitors of protein kinases, staurosporine (45) and H7 (46). In rat hepatocytes, glucagon has been shown to stimulate production of inositol phosphate as well as that of cAMP (39). PMA has also been shown to mimick the effect of glucagon on hepatic steroid metabolism (48). our results showing the inhibitory effect of PMA on IGFBP-1 mRNA levels and the stimulatory effect of glucagon seen in PKC depleted cells rule out the possibility that glucagon may be acting via activation of PKC. Agents that elevate intracellular levels of cAMP, such as IBMX and $(Bu)_2$cAMP were found to stimulate IGFBP-1 mRNA levels in a dose dependent manner. Glucagon appeared to mediate its stimulatory effect on IGFBP-1 mRNA levels by elevating intracellular levels of cAMP as the inhibitors of protein kinases, staurosporine and H7 were found to block this effect of glucagon. Staurosorine and H7 (45,46) have been shown to inhibit PKA as well as PKC, indicating that glucagon may be stimulating IGFBP-1 mRNA levels

through a PKA mediated mechanism. IGFBP-1 production has been shown to be stimulated in vitro in human fetal liver explants by glucagon and by the agents that elevate intacellular levels of cAMP such as theophylline and forskolin (49).

We demontrated that bGH, T_3 and insulin inhibited whereas glucagon and dexamethasone stimulated IGFBP-1 mRNA levels in hepatocytes. Insulin appeared to be the most potent inhibitor and suppressed the effect of dexamethasone or glucagon on IGFBP-1 mRNA levels whereas bGH and T_3 appeared to be weak inhibitors. Glucagon was as potent as dexamethasone in stimulating IGFBP-1 mRNA levels. Glucagon when added in combination with dexamethasone produced an additive effect on IGFBP-1 mRNA stimulation indicating that glucagon and dexamethasone may be acting via different mechanisms to stimulate IGFBP-1 mRNA levels. Insulin has been shown to inhibit hepatic IGFBP-1 mRNA levels in vivo when administered in diabetic rats (30) and in vitro in H4IIE rat hepatoma cells (35). Dexamethasone increased hepatic IGFBP-1 mRNA in vivo in rats (50) and in vitro in H4IIE rat hepatoma cells (44). Effect of bGH, T_3 and glucagon on IGFBP-1 mRNA levels have not previously been reported.

In this study, we have also shown that bGH superinduces IGFBP-1 gene in hepatocytes in the presence of cycloheximide. Estrogen has been shown to superinduce c-fos gene in the presence of cycloheximide (51). Cycloheximide has also been shown to superinduce proenkephalin A mRNA levels in normal B

lymphocytes (52) and c-myc mRNA in bursal lymphoma cells (53). The mecahnisms involved in enhanced gene expression by cycloheximide have not been elucidated, but reports suggest that the inhibition of protein synthesis by cycloheximide can alter mRNA levels through effects on transcription (54) or translation (55).

A common hypothesis to explain the increased transcription of a gene by a protein synthesis inhibitor is the inhibition of a labile protein repressor which is involved in inhibition of gene expression (56). More recently it has been suggested that cycloheximide could contribute to conformational regulation of gene induction by rapid phosphorylation of chromatin associated proteins (57).

IGFBP-1 mRNA in hepatocytes has a short half life of 2 h. bGH has no posttranscriptional effect on IGFBP-1 mRNA stability whereas glucagon or $(Bu)_2$cAMP appear to stabilize IGFBP-1 mRNA at a posttranscriptional level. Whether cAMP has any effect on IGFBP-1 gene transcription is not known. cAMP-responsive elements (CRE) and its associated trans-activators have been implicated in the cAMP regulation of several genes (58). There is no evidence for consensus binding sequences for this CRE, AP-1 or AP-2 on human IGFBP-1 gene (59,60). cAMP has been well documented in stabilization of a number of mRNAs. cAMP stabilizes phosphoenolpyruvate carboxykinase mRNA (61), osteocalcin mRNA (62) and chorionic gonadotropin mRNA (63). The mechanisms by which cAMP stabilizes mRNAs are not known, although mRNA stability is determined by specific sequences

and/or structures in the transcripts (64,65). The other factors responsible for mRNA stability are (a) change in length of poly (A) tail (66), (b) Poly (A) binding protein complexed to poly (A) tail (67) and (c) the presence of destabilizing sequence $(AUUU)_n$ in 3'-Untranslated region (68).

In conclusion, the present study has given an insight into the various hormonal regulators of IGFBP-1 mRNA in rat hepatocytes and the possible mechanisms of their action. Primary cultured rat hepatocytes may constitute an ideal model for studying the molecular mechanisms of hormonal regulation of IGFBP-1 gene expression.

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CHAPTER 7

GENERAL DISCUSSION

SUMMARY

INSULIN LIKE GROWTH FACTOR-1

1. GH and glucagon individually stimulated IGF-I mRNA levels 2.0 to 2.5 fold and 1.8 to 2.0 fold respectively, but produced a synergistic effect and stimulated IGF-I mRNA levels 10-12 fold when combined.
2. The stimulatory effects of GH and glucagon were also observed on IGF-I peptide production in the culture medium. But bGH and glucagon produced an additive effect on IGF-I peptide production when combined.
3. Somatostatin analog, octreotide inhibited hGH induced stimulation of circulating levels of IGF-I in serum and hepatic IGF-I mRNA abundance in hypophysectomized rats. The level of glucagon in serum was found to be reduced when hGH and octreotide were administered in hypophysectomized rats. Octreotide was also found to have a direct effect on inhibition of IGF-I mRNA levels in cultured rat hepatocytes.
4. Glucagon appeared to mediate its stimulatory effect on IGF-I mRNA levels and produced a synergistic effect in the presence of GH via elevation of cAMP levels.
5. bGH appeared to stimulate IGF-I mRNA levels via elevation of PKC levels.
6. Stimulation of PKA and PKC levels is necessary but not sufficient to produce a synergistic effect on stimulation of IGF-I mRNA levels.
7. A synthesis of one or more new protein(s) is required with

elevation of PKA and PKC levels to produce a synergistic effect on stimulation of IGF-I mRNA levels.

8. Insulin and EGF appear to inhibit IGF-I mRNA levels in the presence or absence of bGH, glucagon or bGH plus glucagon.
9. Glucagon and GH appear to be the major regulators of hepatic IGF-I gene expression.

INSULIN LIKE GROWTH FACTOR BINDING PROTEIN-1

1. Glucagon stimulated whereas bGH inhibited IGFBP-1 mRNA levels in hepatocytes. These effects were also observed on IGFBP(s) peptide production by hepatocytes in their culture medium.
2. Glucagon stimulated IGFBP-1 mRNA levels via elevation of cAMP levels, whereas bGH appeared to mediate its effect via elevation of PKC to inhibit IGFBP-1 mRNA levels.
3. A synthesis of one or more protein(s) is required besides stimulation of PKC levels to inhibit IGFBP-1 mRNA levels by GH.
4. In the presence of cycloheximide, bGH superinduced IGFBP-1 mRNA levels in hepatocytes.
5. Insulin is the most powerful inhibitor whereas glucagon and dexamethasone are the most potent stimulators of IGFBP-1 mRNA levels.
6. Glucagon and dexamethasone added together produced an additive effect on IGFBP-1 mRNA stimulation indicating that they act via different mechanisms to stimulate IGFBP-1 mRNA

levels.

7. Insulin inhibited IGFBP-1 mRNA levels in the presence of dexamethasone or glucagon.

DISCUSSION

The purpose of this thesis was to study the hormonal regulation of IGF-I and IGFBP-1 gene expression in cultured rat hepatocytes. Since liver is the major source of production of circulating levels of IGF-I and IGFBPs in the serum, hepatocyte cultures maintained under serum free conditions was found to be the ideal system for our purpose.

Effect of bGH and glucagon on IGF-I mRNA accumulation in rat hepatocytes

As discussed in Chapter 2, bGH and glucagon each showed a modest effect on stimulation IGF-I mRNA levels but produced a synergistic effect on IGF-I mRNA accumulation when combined. bGH and glucagon also stimulated IGF-I secretion in hepatocytes but produced an additive effect when combined.

The importance of glucagon's effect was also noted in vivo in rat. Somatostin analog, octreotide was found to inhibit hGH induced stimulation of circulating levels of IGF-I in serum and also hepatic IGF-I mRNA levels in hypophysectomized rat. Serum levels of glucagon was reduced in hGH plus octreotide administred hypophysectomized rats. Octreotide was also found to inhibit IGF-I mRNA levels in cultured rat hepatocytes. These data indicate that octreotide appears to have an indirect effect on hGH stimulation of IGF-I mRNA levels by inhibiting

serum glucagon levels and it also has a direct inhibitory effect on IGF-I mRNA levels.

The unique synegy observed with bGH and glucagon effect on IGF-I mRNA stimulation was not observed when T_3, oPRL, EGF, insulin and dexamethasone were each combined with bGH or glucagon. These data indicate that the major hormonal regulators of hepatic IGF-I mRNA levels appear to be GH and glucagon.

The mechanism of glucagon and GH effect on IGF-I mRNA accumulation in rat hepatocytes

Glucagon has been shown to stimulate production of inositol phosphate as well as that of cAMP in rat hepatocyte (1). When IBMX or $(Bu)_2$cAMP were combined with bGH, a synergistic effect on IGF-I mRNA stimulation was observed. PMA when added in combination with bGH, failed to produce a synergistic effect on IGF-I mRNA accumulation indicating that glucagon may be acting via elevation of cAMP levels rather than via elevation of PKC. The effect of glucagon and cAMP on IGF-I mRNA stimulation has been observed (2) but no synergistic effect of bGH plus glucagon has been reported before.

Trophic hormones have been shown to stimulate IGF-I production in various cell types. In all instances the stimulation of cAMP production occurs concomitantly. This appears to be true for FSH and LH acting on porcine granulosa cells (3) and ACTH acting on cultured bovine adrenal cells (4), TSH acting on thyroid follicular cells (5) and PTH acting

on osteoblasts (6). In the case of liver, glucagon has been implicatd as a trophic factor for regenerating liver (7). It is possible that many trophic hormones activate both cAMP production and another pathway (i.e. protooncogenes) leading to IGF-I production and growth. This seems to explain the effect of EGF on growth-arrested rat H4IIE hepatoma cells (8).

GH has been shown to stimulate the formation of diacylglycerol (DAG) in canine kidney membrane (9), Ob1771 mouse preadipocytes (10) and in rat hepatocytes (11). This indicates that PKC could be a mediator of GH action. We have shown that PMA when added in combination with glucagon or $(Bu)_2$cAMP do not synergize stimulation of IGF-I mRNA levels. But the effect of bGH plus glucagon on IGF-I mRNA stimulation is inhibited 80 % in PKC depleted cell and 80 to 90 % by the inhibitors of PKC. To investigate if a new protein synthesis was required for bGH plus glucagon effect on IGF-I mRNA stimulation, 10 ug/ml cycloheximide was used to inhibit protein synthesis by 90 %. bGH plus glucagon effect on IGF-I mRNA stimulation was inhibited 85 % in the presence of cycloheximide indicating that the synthesis of one or more protein (s) may be required besides elevation of PKC and PKA levels.

Our results indicate that insulin or EGF inhibit IGF-I mRNA levels in the presence of bGH, glucagon and bGH plus glucagon. Insulin has been reported to stimulate IGF-I mRNA levels in hepatocytes (13). The discrepancy could be due to the

different culture conditions employed. (this is discussed in chapter 3).

Effect of glucagon on IGF-I mRNA stability

Half life of hepatic IGF-I mRNA is 12 h. bGH was found to have no posttranscriptional effect on IGF-I mRNA stability, whereas glucagon and $(Bu)_2$cAMP were found to stabilize IGF-I mRNA at a posttranscriptional level. The mechanism of cAMP on mRNA stability is not known but it is found to stabilize several mRNAs (13).

GH has been shown to stimulate tyrosine kinase activity associated with GH receptor in various cell types (14) and tyrosyl phosphorylation of cellular proteins (15). There may be a possible role of tyrosine kinase activity in action of GH. GH has also been shown to stimulate c-fos and c-jun mRNAs in 3T3-F442A preadipocytes (16). These proteins may be important in mediating GH effect on IGF-I mRNA levels besides activating PKC levels.

The synergistic effect of glucagon and $(Bu)_2$cAMP on IGF-I mRNA levels when added in combination with bGH could be due to upregulation of GH receptors by glucagon (17). The inhibitory effect of insulin and EGF on IGF-I mRNA levels may be due to downregulation of GH receptors in hepatocytes (17). This area needs further investigation with regards to the time required for the synthesis or downregulation of GH receptors.

IGFBP-1 mRNA LEVELS

Effect of bGH and glucagon on IGFBP-1 mRNA levels

Glucagon stimulated whereas GH inhibited IGFBP-1 mRNA levels. The agents that elevate intracellular levels of cAMP also stimulated IGFBP-1 mRNA levels indicating that glucagon may be mediating its effect on IGFBP-1 mRNA accumulation via cAMP pathway. GH appears to inhibit IGFBP-1 mRNA levels via elevation of PKC levels and also appears to require synthesis of one or more portein(s). In the presence of cycloheximide, GH superinduced IGFBP-1 mRNA indicating the presence of a repressor protein which inhibits IGFBP-1 gene transcription.

Effect of various hormones on IGFBP-1 mRNA levels in rat hepatocytes

Insulin is the most powerful inhibitor whereas glucagon and dexamethasone are the most potent stimulators of IGFBP-1 mRNA levels in hepatocytes. T_3 was also found to inhibit IGFBP-1 mRNA levels. Dexamethasone when added with glucagon produced an additive effect on IGFBP-1 mRNA stimulation indicating that glucagon and dexamethasone act via different mechanisms to stimulate IGFBP-1 mRNA levels. Insulin inhibited IGFBP-1 mRNA accumulation in the presence of dexamethasone or glucagon.

Glucagon's effect on IGFBP-1 mRNA stability

Half life of hepatic IGFBP-1 mRNA is 2 h. bGH has no posttranscriptional effect whereas glucagon stabilizes IGFBP-1

mRNA at a posttranscriptional level.

In conclusion, bGH and glucagon appear to be the major regulatory hormones for IGF-I mRNA stimulation in hepatocytes. They appear to act via PKA as well PKC pathways and also require synthesis of one or more protein(s). bGH inhibited whereas glucagon stimulated IGFBP-1 mRNA levels in hepatocytes. bGH appers to act via PKC pathway and requires synthesis of one or more protein(s) whereas glucagon appears to mediate its effect via PKA pathway. The effect of GH on stimulation of IGF-I production observed in vivo could be due to its direct effect on IGF-I gene transcription and also due to inhibition of hepatic IGFBP-1 production. When IGFBP-1 production is reduced, more free IGF-I is released in circulation. Once the proteins required for bGH plus glucagon's effect on IGF-I mRNA accumulation and those requied for bGH effect on IGFBP-1 mRNA levels are identified and characterized, we would have a better understanding of the mechanisms involved in hepatic IGF-I and IGFBP-1 mRNA regulation.

Figure 1 illustrates the schemetic model of the molecular events associated with the mechanisms of glucagon and growth hormone action on IGF-I and IGFBP-1 mRNA levels in cultured rat hepatocytes.

Figure 1

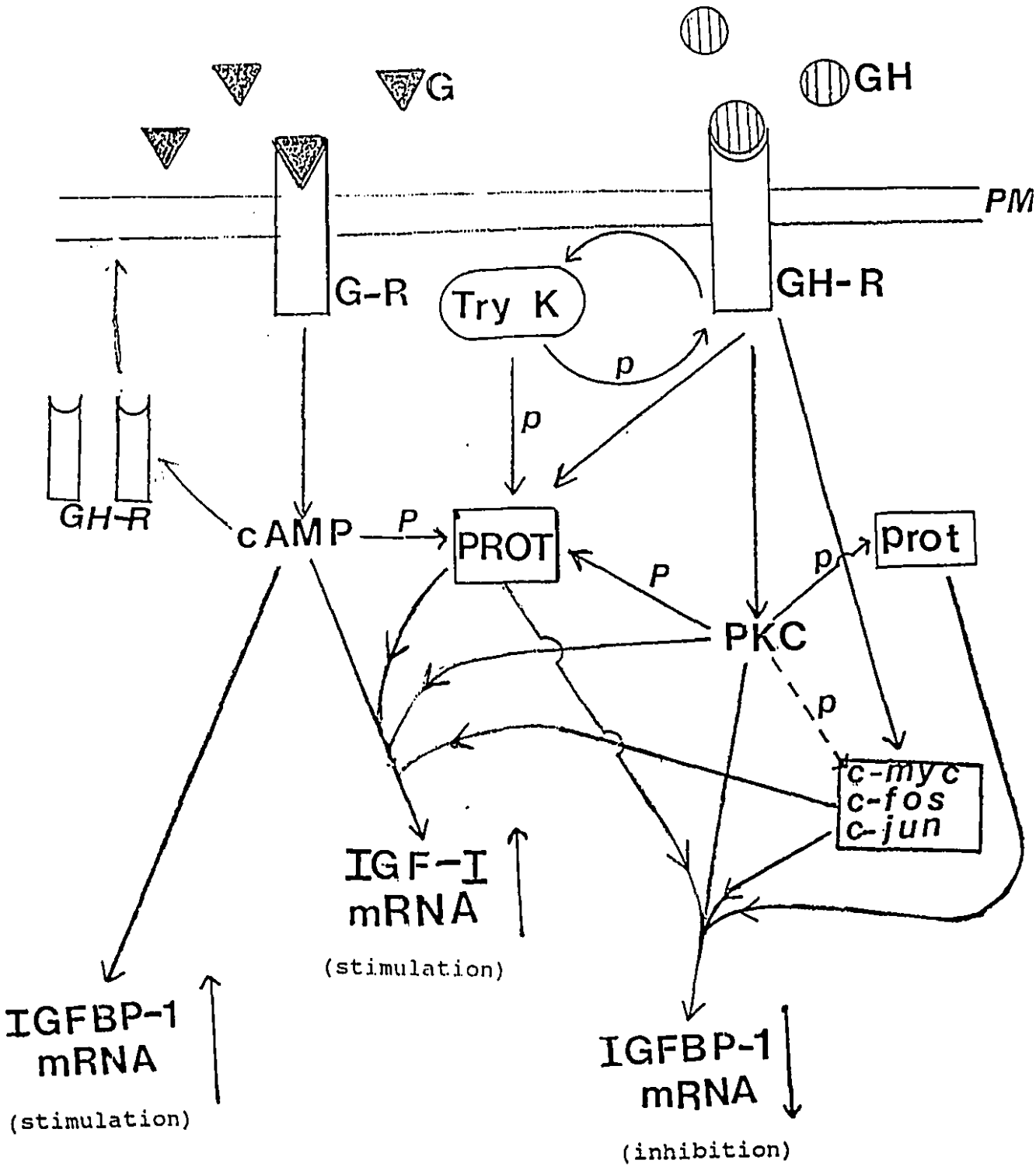
Schemetic model illustrating the molecular events associated with the mechanisms of glucagon and growth hormone action on IGF-I and IGFBP-1 mRNA levels in cultured rat hepatocytes

Glucagon (G) acting via glucagon receptor (G-R) may elevate cAMP levels which modestly stimulates IGF-I mRNA levels in hepatocytes. Glucagon also upregulates GH receptors in hepatocytes via cAMP pathway. Growth hormone (GH) acting via growth hormone receptor (GH-R) appears to stimulate PKC and it may also activate synthesis of c-myc, c-fos and c-jun oncoproteins as well as other proteins. GH also stimulates tyrosine kinase activity which phosphorylates GH-R and may also phosphorylate one or more proteins whose synthesis is stimulated by GH. cAMP and PKC may also be involved in phosphorylating one or more protein synthesised by GH. Glucagon and GH's synergistic effect on IGF-I mRNA stimulation appears to require PKC, cAMP as well as synthesis of one or more proteins.

Glucagon stimulates cAMP levels which may stimulate IGFBP-1 mRNA levels in hepatocytes. GH may stimulate PKC as well as synthesis of certain proteins. PKC in the presence of one or more proteins appears to inhibit IGFBP-1 mRNA levels in hepatocytes.

Figure 1

Schemetic model illustrating the molecular events associated with the mechanisms of glucagon and growth hormone action on IGF-I and IGFBP-1 mRNA levels in cultured rat hepatocytes



CONTRIBUTION TO KNOWLEDGE

Insulin-like growth factor-I

The effect of GH on stimulation of hepatic IGF-I mRNA has been shown in vivo in rats (18) and in vitro in hepatocytes (12,19).

We have also shown that glucagon or cAMP modulates GH effect and produces a synergistic effect on IGF-I mRNA stimulation and an additive effect on hepatic IGF-I production (19). This may have some clinical implications in some pathophysiological conditions where GH levels are normal but IGF-I levels are either elevated or low. Knowing that glucagon may be playing an important role in hepatic production of IGF-I, it may be helpful in management of the patient with low serum level of IGF-I. The augmentation by glucagon of GH effect on IGF-I production has not been reported previously.

It is generally accepted that insulin stimulates hepatic IGF-I production and hepatic IGF-I gene expression in streptozotocin induced diabetic rats (20). Serum IGF-I levels are low in diabetic patients and reach normal levels by insulin treatment (21). IGF-I production is stimulated by insulin in hepatocytes prepared from normal rats and from insulin treated diabetc rats (22), but IGF-I production is not stimulated in hepatocytes prepared from diabetic rats (22). This indicates that there may be other factors involved in producing insulin's stimulatory effect on hepatic IGF-I production. Insulin has

been shown to stimulate hepatic IGF-I gene exression in vitro in rat hepatocytes (12). But we find that insulin has an inhibitory effect on IGF-I gene expression in hepatocytes. Insulin's effect on stimulation of IGF-I gene expression observed in vivo may be an indirect effect and it may be a result of interaction of various hormones.

In protein deprived rats, hepatic IGF-I mRNA levels are low and reach normal levels after refeeding the rats normal diet (23). The mechanism of these effect on protein deprivation on hepatic IGF-I mRNA levels is not known. Our results show that in the presence of cycloheximide, GH plus glucagon fail to stimulate IGF-I mRNA levels indicating that synthesis of one or more proteins induced by GH may be essential in stimulating IGF-I gene expression by glucagon plus GH.

INSULIN LIKE GROWTH FACTOR BINDING PROTEIN-1

Controversial results have been reported on GH effect on IGFBP-1 mRNA levels in hypophysectomized rats. In hypophysectomized rats, hepatic IGFBP-1 mRNA levels were elevated in one study, and reached the control level by GH treatment (24), whereas in the other, GH appears to have no effect on elevated IGFBP-1 mRNA levels in hypophysectomized rats (25). We have shown for the first time in vitro that GH inhibits IGFBP-1 mRNA levels in hepatocytes.

In protein deprived or fasted rats, IGFBP-1 mRNA levels are elevated and they reach normal levels after refeeding (26). We have observed that in the presence of cycloheximide, GH

superinduces IGFBP-1 mRNA levels. Again this may be true for the other hormones which inhibit IGFBP-1 mRNA levels under normal conditions.

Effect of thyroid hormone on IGFBP-1 mRNA levels has not been previously reported. We have shown that in vitro T_3 inhibits IGFBP-1 mRNA levels in hepatocytes.

Though we have shown the effect of various hormones on stimulation or inhibition of IGF-I and IGFBP-1 gene expression, we presume that these data will reflect the same effects on the production of these peptides in the culture medium.

Effect of various hormones on expression of IGF-I and IGFBP-1 mRNA levels in rat hepatocytes

Some hormones that inhibit IGFBP-1 mRNA levels appear to stimulate IGF-I and IGFBP-3 mRNA levels. GH, T_3 and insulin stimulate hepatic IGF-I mRNA levels in vivo, whereas they inhibit IGFBP-1 mRNA levels in hepatocytes and increase IGFBP-3 mRNA levels. This may be one way of stabilizing IGF-I by increasing its half life and regulating its availability to the various tissues. When IGFBP-3 levels are elevated at the same time as IGF-I levels, IGF-I bound to IGFBP-3 protects the organism from hypoglycemic effect of IGF-I and free IGF-I is released as required.

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