Regulation of Insulin-like Growth Factor-I and -II by Glucose in Primary Cultures of Fetal Rat Hepatocytes*

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A selective primary culture of fetal rat hepatocytes was established in our laboratory in order to elucidate the molecular mechanisms of action of different factors and conditions on insulin-like growth factor (IGF)-I and -II gene expression during the perinatal period of the rat. In this model we report that, in a serum-free condition and in the presence of non-stimulatory doses of insulin, 5–20 mM glucose evoked an increase of IGF-I and -II mRNA abundance. Glucose regulated in a parallel manner IGF peptide secretion, and an excellent correlation was observed between IGF-I and -II mRNA and IGF-I and -II peptide levels in the conditioned media in response to the carbohydrate. The experiment with 2-deoxyglucose suggests that glucose 6-phosphate, but not its further metabolism, is necessary for the induction of IGF transcript abundance in cultured fetal hepatocytes. Finally, the glucose-induced rise in IGF-II mRNA, the main IGF in fetal stages, was mediated by stimulation of gene transcription and increased transcript stability. The results support the idea that IGFs belong to a family of genes that are positively regulated by glucose.

Insulin-like growth factors (IGFs)1-1 and -II are cell growth regulators that originate largely in the liver (1). Hepatic production of IGFs appears to be regulated at pretranslational levels, as indicated by the strong correlations between circulating IGFs and abundance of hepatic IGF-I and -II mRNA (2–4). Secretion of both IGFs in adult animals is mainly regulated by growth hormone (GH), but nutritional status and serum insulin concentration are important factors involved in the regulation of IGF synthesis and secretion (1–4). Previous work “in vivo” has shown that a balanced insulin/nutrients ratio regulates IGFs secretion during the perinatal period of the rat, when the IGF response to GH is not yet well established (2). We have recently demonstrated that refeeding and insulin treatment of undernourished and diabetic neonatal rats, respectively, lead to a recovery of serum and liver mRNA expression of IGF-I and -II without a prior increase in serum GH (3). These in vivo experiments strongly suggest that during the perinatal period of the rat, IGFs regulation is GH-independent, supporting the role of insulin and nutrients. However, in experiments in vivo the simultaneous fluctuations of fuels and hormones that occur in diabetic and undernourished animals make it difficult to demonstrate specific regulation. It seemed appropriate, therefore, to investigate “in vitro” the underlying mechanisms for IGFs regulation. The in vitro system that most closely resembles normal developing liver is the primary culture of fetal hepatocytes (5–7). Since IGF expression in primary fetal cultures is limited and subject to plastic substrate-induced changes in the differentiation state of the liver cells, very scant data are available in the literature about the IGF response of fetal hepatocytes in culture to different conditions (8–11). To overcome these difficulties, a selective primary culture of fetal rat hepatocytes was established in our laboratory in order to show unequivocally the specific effect of different factors and conditions on IGF-I and -II gene expression during the mammalian perinatal period, as well as to elucidate their molecular mechanism of action. Gene expression, determined by the highly sensitive RNase protection assay, and peptide secretion of IGF-I and -II were assayed in cultures of hepatocytes from rat fetuses on day 21 of gestation in the presence of serum, GH, and glucose. In this model we report that fetal rat hepatocytes retain some of the characteristics of the rat fetal liver while maintained in short term culture, i.e. rat fetal hepatocytes synthesize and secrete IGFs to the culture medium. The results also demonstrate, for the first time, the specific regulatory role of glucose on IGF-I and -II gene expression during late fetal stages of perinatal development. This system should be a useful tool for further studies of molecular mechanisms of IGF-I and -II regulation.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human IGF-I and -II (Roche Molecular Biochemicals) were used as standard and for iodination. RNase A and RNase T1 were also purchased from Roche Molecular Biochemicals. Na125I and Hyperfilm-MP autoradiography film were obtained from Amersham Pharmacia Biotech. Polyclonal antiserum (lot K9147-48) raised in rabbit against human IGF-I and the C-terminal fragment (residues 57–70) was purchased from KabiGen AB (Stockholm, Sweden). [32P]UTP was purchased from ICN (Nuclear Ibérica S.A., Madrid, Spain). Riboprobes Gemini II Core System (Promega Corp., Madison, WI) was used for the generation of RNA probes. Cycloheximide, actinomycin D, and bovine serum albumin were purchased from Sigma. Doxorubicin hydrochloride was purchased from Aldrich. Fetal calf serum (FCS), medium 199 (M199), and Dulbecco's modified Eagle's medium were purchased from BioWhittaker (Ingelheim Diagnostica y Tecnologia, Madrid, Spain).
Experimental Models

Wistar rats bred in our laboratory with controlled temperature and artificial dark-light cycle were used throughout the study. Females were caged with males and mating was confirmed by the presence of spermatozoa in a vaginal smear. Each dam was housed individually from the 14th day of pregnancy. Animals were fed a standard laboratory diet ad libitum. Water was given ad libitum. Dams were sacrificed and fetuses were exposed after abdominal incision. All experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for care and use of experimental animals (Bethesda).

Cell Extraction

Fetal Hepatocytes—Primary cultures of hepatocytes from 21-day-old Wistar rat fetuses were prepared by a non-perfusion collagenase dispersion method (5). The protocol involves incubation of the minced tissue in Krebs bicarbonate buffer containing 0.5 mM EGTA in a 150-ml conical flask for 30 min at 37 °C in a shaking water bath (100 cycles/min) under continuous gassing. After 60 min, the cell suspension was centrifuged at 50 × g for 5 min, and the supernatant was discarded. Cells were then resuspended in Krebs bicarbonate buffer containing 2.5 mM Ca2+ and 0.5 mg of collagenase/ml in a 150-ml conical flask. The mixture was incubated at 37 °C in a shaking water bath (100 cycles/min) under continuous gassing. After 60 min, the cell suspension was washed with Krebs bicarbonate buffer containing 2.5 mM Ca2+ and then centrifuged at 35 × g for 5 min and filtered through a nylon mesh (500 μm). The washing step was repeated with a nylon mesh of 100 μm. During washings at very low speed and separation occurred between parenchymal and hematopoietic cells, the latter mostly remaining in suspension. By counting under a microscope, hematopoietic cell contamination was shown to be lower than 5%. The procedure produced ~1.5 × 106 cells/g of fetal liver, representing about a 15% recovery yield. Cell viability (trypan blue exclusion) for fetal hepatocytes was always higher than 95%.

Adult Hepatocytes—Isolation of adult hepatocytes was carried out from 3-month-old male rats by perfusion with collagenase in Krebs/bicarbonate buffer under continuous gassing. After 60 min, the cell suspension was washed twice with sterile Dulbecco’s modified Eagle’s medium and then resuspended in this medium supplemented with 50 μg/ml gentamicin, 50 μg/ml penicillin G, and 50 μg/ml streptomycin.

Cell Culture

Fetal Hepatocytes—For culture of fetal cells sterile techniques were used throughout the procedure, and media were supplemented with 120 μg of penicillin–O/ml, 100 μg streptomycin/ml. The isolated cells were plated in 100-mm diameter plastic dishes containing 8 ml of medium 199 with Earle’s salts supplemented with 10% (v/v) fetal calf serum and antibiotics as described above. Each dish was inoculated with 6 × 106 cells, and the primary culture was kept at 37 °C under an atmosphere of 5% CO2 in air with 80% humidity in a cell incubator for 4 h. Then the attached monolayer of cells was washed with serum-free medium, and fresh medium was added supplemented with the various different conditions was added and the dishes incubated either for 3 or 16 h. The use of this procedure ensures a fairly pure culture of fetal hepatocytes in which the fibroblast-like cells comprise less than 10% of the total cells (6).

Adult Hepatocytes—3 × 106 hepatocytes were plated in 100-mm diameter tissue culture dishes in a medium containing 8 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. After 4 h incubation to facilitate cell attachment to the matrix, the medium was aspirated, and the plates were washed twice with phosphate-buffered saline to remove the nonadherent cells and filled with 8 ml of Dulbecco’s modified Eagle’s medium lacking serum. Additions were made so that the changes in the total incubation volume were less than 2%.

Iodination, Purification, and Determination of IGF-I and IGF-II

Recombinant human IGF-I and IGF-II were labeled by a modified chloramine-T method (2, 3). The specific activity achieved with this method was approximately 90–175 μCi/μg for both peptides. Prior to IGF-I and -II determination, culture medium was concentrated, and serum IGFBPs were removed by standard acid gel filtration. This method has proved to be the most reliable one for use with rat serum (2, 3).

The radioimmunoassay (RIA) for IGF-I and rat liver membrane receptor assay (RRA) for IGF-II were carried out as described previously (2, 3). The coefficients of variation within assay and between assays were 8.0 and 12.4%, respectively.

Preparation of RNA

Total RNA—Cultured hepatocytes were separated from the plastic substrate with a rubber policeman, and total RNA was prepared by homogenization of cells in guanidinium thiocyanate as originally described (12). RNA was re-precipitated for purification, and its concentration was determined by absorbance at 260 nm. Samples were electrophoresed through 1.1% agarose, 2.2 mol of formaldehyde/liter gels and stained with ethidium bromide in order to render the 28 S and 18 S ribosomal RNA-visible and thereby confirm the integrity of the RNA and normalize the quantity of RNA in the different lanes. A β-actin probe (0.6-kilobase pair EcoRI/HindIII fragment isolated from the VC18 vector kindly provided by Dr. P. Martin-Sanz from Instituto de Bioquímica, CSIC, Madrid, Spain) was used in a Northern blot assay in order to validate the ethidium bromide method for loading normalization.

Nuclear-enriched RNA—Nuclear pellets were extracted from 10 × 106 cells. Hepatocytes were removed from the plates with a rubber policeman in phosphate-buffered saline and centrifuged at 15,000 rpm for 15 s. Cell pellet was resuspended in 10 ml HEPES-KOH, pH 7.9, at 4 °C, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, allowed to swell on ice for 15 min, and then vortexed for 10 s. Samples were centrifuged for 15 s at 15,000 rpm, and the nuclear pellets were resuspended on ice in a solution containing 4 μM guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. Purification, precipitation, and quantification of the nuclear-enriched RNA were as described above for total RNA.

Riboprobes

Rat IGF-I, IGF-II, and IGFBP3 cDNAs were kindly provided by Dr. E. Hernández (Instituto de Bioquímica, CSIC, Madrid, Spain), Dr. C. T. Roberts, and Dr. D. LeRoith (National Institutes of Health, Bethesda). Rat IGF-I cDNA ligated into a PGem-3 plasmid (Promega Biotech, Madison, WI) was linearized with HindIII, and an antisense riboprobe was produced by T7 RNA polymerase generating two protected fragments of 224 bases (1a) and 386 bases (1b). Rat IGF-II cDNA ligated into a PGem-3 plasmid was linearized with HindIII and incubated with T7 RNA polymerase to generate a riboprobe that recognized a protected fragment of 500 bases. Rat IGFBP3 cDNA ligated into a PGem-4Z plasmid was linearized with AciI, and use was made of T7 RNA polymerase to generate a 343-base-long antisense riboprobe.

Solution Hybridization/RNAse Protection Assay (RPA)

Solution hybridization/RNase protection assays were performed as described previously (13). Briefly, 20 μg of total liver RNA were hybridized with 500,000 cpm of the 32P-labeled riboprobes described above for 18 h at 45 °C in 75% formamide and 400 mmol of NaCl/μl. After RNase digestion with a buffer containing 40 μg of RNase A/ml and 2 μg of RNase T1/ml for 1 h at 37 °C, protected RNA-RNA hybrids were resolved on denaturing 5% polyacrylamide and 8 mol of urea/liter gels. Autoradiography was performed at ~70 °C against an Hyperfilm MP film between intensifying screens. Bands representing protected probe fragments were quantified using a Molecular Dynamics scanning densitometer and accompanying software.

Analysis of mRNA Stability

Fetal hepatocytes were cultured with 0.01 μM insulin in the absence or presence of 20 μl glucose for 16 h. Then at time 0, 1 μg/ml doxorubicin and 0.1 μM insulin were added to fresh medium with or without 20 μl glucose and cultures stopped at different time points. Total RNA was processed as above, and IGF-I and -II mRNA expression was determined by RPA.

Statistical Analysis

Data are presented as means ± S.D. Statistical comparisons were performed by one-way analysis of variance, followed by the protected least significant difference test (9).

RESULTS

Basic Conditions for the Culture of Fetal Hepatocytes

Effect of Serum and Glucose Alone on IGF-I and -II Gene Expression—In order to establish the basic conditions of the
fetal hepatocyte in culture, the response of IGF-I and -II gene expression to 10% fetal calf serum (FCS), glucose, or stimulating doses of GH in the medium was evaluated. Fetal hepatocytes cultured in the presence of FCS-free plain essential M199 medium were used as untreated controls (Fig. 1A, column C). Treatment with 10% FCS for 16 h provoked an increase in the abundance of IGF-I and -II mRNA transcripts (Fig. 1A, column S), an effect directly dependent on serum factors and not on the amount of protein present in the medium as demonstrated by the lack of effect of an equivalent amount of bovine serum albumin (Fig. 1A, column A). In a time course response experiment, increased IGF-I and -II mRNA expression induced by 16 h treatment with 10% FCS (time 0) decayed faster in FCS-free conditions (Fig. 1A, serum columns in the right panel) than in the presence of 10% FCS (Fig. 1A, + serum columns in same panel) which maintained higher levels of both transcripts at 9 and 16 h of culture. Therefore, in order to avoid the stimulating effect of serum, the remaining experiments were carried out in the absence of serum, and the cell culture viability in the absence of serum was ensured for at least 24 h. No significant effect on IGF-I and -II was observed when fetal hepatocytes were treated with increasing concentrations of glucose (0–20 mM) added to the serum-free medium in the absence of any other factor (Fig. 1B). In all experiments, at similar time exposures of films, IGF-II transcript abundance in fetal hepatocytes was at least 5-fold higher than that of IGF-I, in agreement with the fact that IGF-II is reputed to be the major IGF in fetal stages.

**Effect of Growth Hormone on IGF-I and -II Gene Expression**—The IGF-I and -II mRNA response of fetal hepatocytes to increasing doses of GH was assayed and compared with the response of adult hepatocytes in order to test GH as a regulatory factor of IGFs during the fetal period (Fig. 2). As expected, no induction of IGF-I and -II gene expression was observed in cultures of fetal hepatocytes treated for 16 h with different doses of GH (5–50 ng/ml) (Fig. 2A); however, the same GH concentrations evoked a significant increase of IGF-I and IGFBP3 gene expression in cultures of adult hepatocytes (Fig. 2B), supporting the idea that factors other than GH might regulate IGFs gene expression at fetal stages.

**Effect of Glucose Plus Insulin on IGF-I and IGF-II Gene Expression and Peptide Secretion in Fetal Hepatocytes**

Gene Expression—Although glucose per se had no significant effect on IGF gene expression, when a minimal non-stimulating dose of 0.01 μM insulin was added to the medium together with the same concentrations of glucose, a significant increase in the abundance of transcripts of IGF-I and -II was observed both at short (3 h) and long (16 h) term treatments (Fig. 3). The glucose-induced stimulation was more evident for IGF-II than for IGF-I in all cases (optical density units in densitometric figures do not represent actual values since arbitrary units were averaged from different films with distinct time exposures). A gradual dose-response increase was observed for IGF-II at 3 and 16 h and for IGF-I at 16 h, while 20 mM glucose evoked the greatest increase of IGF-I and -II mRNA transcripts at both time points. Moreover, in a time course experiment, 20 mM glucose stimulated mRNA expression as soon as 30 min for IGF-II and 1 h for IGF-I after the onset of treatment and remained significantly elevated throughout the experiment (Fig. 4). Similar doses of glucose and insulin had no effect on IGF-I gene expression in cultures of adult hepatocytes (data not shown).

Peptide Secretion—Fetal hepatocytes in culture secrete IGF-I and -II peptides to the medium in response to glucose (Table I). In order to evaluate the IGF peptide secretion, fetal hepatocytes were treated with increasing doses of glucose for 16 h or with 20 mM glucose for different times, and the conditioned medium was assayed for IGF-I (radioimmunoassay) and IGF-II (radioimmunoprecipitation assay). A slight but significant dose-response increase in IGF-I and -II peptide levels in the medium was found at 10 and 20 mM glucose. Besides, a time-dependent increase in IGF-I and -II peptide levels in the medium was observed starting 30 min after the onset of the treatment with 20 mM glucose (Table I). IGF-II values in the conditioned medium were ~10-fold higher than those of IGF-I, which agrees with the higher mRNA expression of IGF-II in these fetal hepatocyte cultures.

When IGF peptide levels (expressed in ng/ml) were correlated with IGF mRNA transcript abundance (expressed in arbitrary units of optical density of RNA bands) obtained from the glucose dose-response experiments, a good correlation was found for both IGF-I (r = 0.976) and -II (r = 0.923) (Fig. 5).

**Mechanism of Action of Glucose on IGF-I and -II Gene Expression**

Characterization of the IGF Response to Glucose—As a first approach to investigate the mechanism of action of glucose on IGF mRNA expression in fetal hepatocytes the following experiments were carried out. Incubation with 10 μg/ml cycloheximide for 16 h, a protein synthesis inhibitor, did not prevent but did reduce the glucose-induced mRNA levels of both IGF-I and -II (Fig. 6A). Incubation with 10 μg/ml of the RNA synthesis inhibitor actinomycin D for 16 h did not change the transcript levels of IGF-I and -II (Fig. 6A). Two different glucose analogs,
2-deoxyglucose (2-DOG) and 3-O-methylglucose (3-OMG), were used to investigate the intracellular pathway where glucose stimulates IGF gene expression. 2-DOG is capable of being metabolized to 2-deoxyglucose 6-phosphate but no further, whereas 3-OMG cannot undergo phosphorylation. A 16-h incubation with doses of 2-DOG, similar to those of glucose previously used, induced a significant increase in IGF-II mRNA transcript abundance and a slight increase in IGF-I (Fig. 6B).

However, incubation of the cultures with the same doses of 3-OMG for 16 h evoked no change in either transcript (Fig. 6B).

**Level of Action of Glucose on IGF Gene Expression**—Transcript stability and transcriptional activity were assayed to delineate the level of action of glucose on IGF-I and -II gene expression. Transcript stability was measured in decay experiments by inducing IGF mRNA transcripts with 20 mM glucose for 16 h and then adding 1 μg/ml doxorubicin at time 0; mRNA was extracted after different times in the absence (—glucose) or presence (+glucose) of 20 mM glucose, and the results are shown in Fig. 7. Increases in IGF-I and -II transcript stability were observed during the 1st h of treatment with doxorubicin versus treatment with the RNA synthesis inhibitor plus glucose, but from that time on the decrease of IGF-I and -II transcripts was faster in the absence than in the presence of glucose. Statistical differences between levels of mRNA abundance in the presence and absence of glucose were found at 0, 0.5, 2, 4, and 6 h for IGF-I and at all times for IGF-II. The transcriptional effect of glucose was determined by the RNase protection assay of nuclear transcripts, a method reported to yield fairly accurate transcriptional activity (see “Discussion”). As a control test, stimulating doses of insulin provoked a large increase in IGF-I nuclear transcripts in cultures of adult hepatocytes (Fig. 8A). The use of this method revealed an increase of IGF-II gene transcription when fetal hepatocytes were treated with 20 mM glucose for 3 h, whereas no significant changes in
nuclear transcripts were observed when treated with 5 and 10 mM glucose (Fig. 8B). Finally, no significant effect of glucose was observed in IGF-I under similar conditions (Fig. 8B).

DISCUSSION

Experimental models in vivo have been widely used for the study of IGF regulation and have shown the important contribution of factors such as nutrients and insulin involved in liver IGF synthesis and secretion (1–3). Although the factors involved in the IGF regulation seem to be the same during the lifetime, the relative contribution is different depending on the stage of development (14). The nature of the IGF regulation is particularly interesting in stages of immaturity when such regulation is GH-independent, and other factors may play a decisive role. However, the study of the molecular pathways and mechanism of action of the different factors on the IGF regulation has been mainly carried out in cultures of adult hepatocytes, which have been shown to produce IGF-I and its binding proteins (15, 16). Moreover, in such cultures, insulin (17–19), GH (20), glucocorticoids (19–21), and amino acid availability (21–23) have been widely reported to regulate IGF-I gene expression. However, the research on IGF and IGFBP synthesis and secretion by fetal hepatocyte cultures is scant mainly due to changes in the differentiating patterns and to the very low synthesis of these peptides by fetal hepatocytes in plastic substratum. The primary culture of late fetal rat hepatocytes described in this article is the first attempt to delineate direct from indirect nonspecific effects of factors, such as insulin and glucose, independently from the effects of other hormones, on the regulation of liver mRNA synthesis of IGFs during stages of development. As already described for fetal stages of rat development, gene expression and peptide synthesis of IGF-II were greater than those of IGF-I (1–3), supporting both the reliability of the model and the main role of IGF-II in fetal growth and differentiation.

The presence of 10% fetal calf serum in the culture medium evoked a rapid increase of IGF-I and -II gene expression in fetal hepatocytes that remained throughout time. Serum contains a number of hormones and growth factors that might stimulate IGF-I and -II gene expression; thus, in order to investigate the specific effect of the different regulatory factors, fetal hepatocytes had to be cultured in serum-free conditions. Physiological concentrations of glucose are present in most of the tissue culture media, but the presence of several doses of glucose (5–20 mM) alone in plain glucose-free media showed no significant effect on IGF-I or -II gene expression in fetal hepatocytes. In order to discard GH as a regulatory factor for IGFs in vitro during the perinatal period, cultures of fetal and adult hepatocytes were treated with GH and the results compared. In contrast with the consistent GH-induced stimulation of IGF-I and IGFBP3 transcripts in cultures of adult hepatocytes, GH evoked no significant changes of IGFs gene expression in fetal cell cultures, supporting the negligible role of GH on IGF regulation at stages of development already demonstrated in vivo by us (2, 3) and other authors (1, 24).

Primary cultures of adult hepatocytes are an excellent model to investigate the specific role of insulin and glucose as these cells are highly sensitive to insulin and survive well in the absence of carbohydrates because of their efficient glucose metabolism (25). Since gluconeogenesis is inefficient at fetal stages, fetal hepatocytes were cultured for a maximum of 24 h, when control hepatocytes cultured in a glucose-free medium continued to show a high viability. Contrary to the significant response to GH described above, adult hepatocytes in culture showed no response of IGFs to increasing doses of glucose in the presence of a minimal non-stimulatory dose of insulin (0.01 μM) in the medium. However, in our cultures of fetal hepatocytes, the presence of 0.01 mM insulin evoked a dose response of IGF-I and -II mRNA expression to glucose. This result agrees with previous studies showing that glucose regulates the gene expression of glycolytic and lipogenic related proteins (25–28), an effect that also requires the presence of insulin in the tissue culture medium (29, 30). Since glucose access to liver cells is insulin-independent, the permissive role played by insulin in the regulation of IGF synthesis by glucose in fetal hepatocyte cultures must be placed at the level of glucose phosphorylation. In contrast to the other hexokinase enzymes, glucokinase is accurately regulated at the pretranslational level by insulin (29), and in liver and cultured hepatocytes, transcription rate of the glucokinase gene is quickly activated by insulin regardless of the glucose concentration (29). Increased glucose metabolism is thought to produce the intracellular signal in the regulatory pathway (31), and although the nature of the signal remains unknown, several possibilities have been proposed (31–33). In order to investigate this intracellular signal, our cultures of fetal hepatocytes were treated with glucose analogs such as 2-DG and 3-OMG. The former is capable of being metabolized to 2-deoxyglucose 6-phosphate but no further, whereas the latter cannot undergo phosphorylation. It has been reported that primary hepatocytes in culture do not respond to 2-deoxyglucose due to high glucose-6-phosphatase activities in these cells, which would rapidly deplete intracellular pools of phosphorylated intermediates (31). However, this non-metabolizable glucose analog can be used in fetal hepatocytes in culture, since glucose-6-phosphatase activity is basically non-existent in fetal stages, and assayable activity is observed several hours after birth (34). Thus, doses of 2-DG similar to those of glucose induced an increase of IGF-II gene expression in the fetal hepatocytes, although less efficient than with glucose, also of IGF-I. Since 3-OMG had an effect on IGF-I and -II gene expression, this would suggest that the hexose 6-phosphate, but not its further metabolism, is necessary for the induction of IGF transcript abundance in cultured fetal hepatocytes. These results agree with those of other au-
thors (31–33, 35) that have proposed the hexose 6-phosphate as an intracellular signal, mediating gene regulation by glucose for other genes in different cell types.

In order to investigate whether physiological doses of glucose in cultures of fetal hepatocytes regulate not only IGF transcript abundance but, in a parallel manner, IGF peptide secretion, IGFs were determined in the culture medium. An excellent correlation was observed between IGF-I and -II mRNA and IGF-I and -II peptide levels in the conditioned media in response to different doses of glucose, suggesting that all steps of the synthetic and secretory pathway are fully functional in the hepatocyte at these immature stages of development.

Since transcript overexpression might result from transcription induction, transcript stabilization, or both, we tested the two possibilities. Transcript stability was determined by transcript abundance decay assays in the presence of the transcription inhibitor doxorubicin. The results showed that IGF-I and IGF-II mRNA transcript stability was higher in the presence than in the absence of glucose at all time points. Increases in IGF-I and -II mRNA both in the absence and presence of glucose were observed during the 1st h of doxorubicin treatment. This paradox could be explained by the simultaneous nonspecific mRNA stabilizing effect of doxorubicin as the result of its intercalation into the 3'-untranslated region of the mRNA and the more specific mRNA de-stabilizing effect of glucose starvation. In this case, the latter would be dominant over the former after the 1st h of treatment. Thus, the glucose-induced rise in

| Table I | 16-h dose response and time course effect of glucose on IGF-I and -II peptide secretion to the conditioned media |
|---------|---------------------------------------------------------------|
| IGF-I (ng/ml) | 0 | 5 | 10 | 20 |
| 11.3 ± 0.1 | 12.4 ± 0.3 | 14.9 ± 1.2 | 17.0 ± 0.7 |
| IGF-II (ng/ml) | 87.5 ± 15.1 | 120.2 ± 21.2 | 154.7 ± 30.0 | 197.5 ± 8.2 |

| Time course (h) | 0 | 1/2 | 1 | 2 | 4 | 8 |
|-----------------|---|-----|---|---|---|---|
| IGF-I (ng/ml) | 9.8 ± 1.3 | 12.4 ± 0.2 | 14.1 ± 0.6 | 14.4 ± 0.8 | 16.5 ± 2.1 | 18.5 ± 5.6 |
| IGF-II (ng/ml) | 57.3 ± 5.2 | 114.0 ± 21.0 | 115.0 ± 2.7 | 169.3 ± 44.0 | 182.0 ± 10.8 | 206.0 ± 26.5 |

a p < 0.05 relative to 0 dose or 0 time.
b p < 0.05 relative to the prior dose or period.
IGF-I and -II mRNA abundance could be partly explained by an increase in transcript stability. Recent work has pointed to RNase protection assay of nuclear transcripts as a reliable method to assess transcriptional activity in cultured cells (36). The increase in transcript content of IGF-II observed by RPA of nuclear transcripts in fetal hepatocytes in response to glucose strongly suggests transcriptional regulation by the carbohydrate. Similar glucose response elements (37) also referred to as carbohydrate response elements (38) have been described in the upstream region or introns of genes that confer a transcriptional response to glucose (37). Our results agree with a transcriptional effect of glucose on IGF-II gene in fetal hepatocytes, as described for the same carbohydrate on other genes (25–28, 31). However, treatment with actinomycin D for 16 h did not prevent the IGF-II response to glucose, but this transcription inhibitor is known to evoke unspecific increases of mRNA transcript content of genes that could partly overcome the overall inhibitory effect on gene transcription. Finally, the inhibitory effect of the protein synthesis inhibitor cycloheximide suggests the need of other protein factors induced by glucose for a complete effect of the carbohydrate on IGF gene expression. Further investigation is needed to expose the protein factor(s) required for the mechanism of action of glucose.

Recently, glucose has been reported to stimulate IGF-I gene expression in C6 glioma cells indicating that IGF-I belongs to a family of genes that are positively regulated by glucose (39) such as L-type pyruvate kinase, insulin, S14, and transforming growth factor-α (25, 31, 39). Our results on IGFs support this positive regulation by glucose and show, for the first time, a transcriptional stimulation of IGF-II by glucose in primary cultures of fetal hepatocytes. Responsiveness of the hepatocyte IGF system to glucose decreases during development since no IGF response to physiological doses of glucose in adult hepatocytes was found. Specific mechanisms have evolved to allow unicellular organisms to metabolize various fuels from the external milieu. These mechanisms involve transcription of genes encoding enzymes of specific metabolic pathways in the presence of appropriate nutrients. In multicellular organisms, especially in mammals, the task of interpreting the environmental changes is handled by hormonal and neuronal pathways. But, perhaps as an evolutionary remnant, nutritional and metabolic signals also play an important role in the regulation of gene expression in multicellular organisms (31). A good example for this dual regulation, fuels and hormones, in the same organism depending on the stage of development is reported in this article, the regulation of IGFs in the developing liver by an essential nutrient such as glucose.

In summary, the results show that isolated fetal rat hepatocytes retain some of the characteristics of the rat fetal liver while maintained in short term culture, making this a reliable model to study specific effects and molecular mechanisms when studying IGF regulation in perinatal stages. In agreement with previous studies in live animals (2, 3), the data demonstrate that IGFs are regulated by glucose at fetal stages rather than by GH which regulates IGFs at adulthood. Glucose shows a dual effect on IGF gene expression, by inducing gene transcription and by increasing transcript stability. Finally, these results have established three immediate goals already in progress in our laboratory as follows: (a) to study the molecular mechanism of action of insulin, glucocorticoids, and other putative regulatory factors on IGF-I and -II synthesis; (b) to confirm the presence of a functional glucose response element/carbohydrate response elements in the IGF-II promoter and characterize its regulation; and (c) to investigate IGFBPs regulation on fetal hepatocyte cultures.
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