INSULIN-LIKE GROWTH-FACTOR BINDING PROTEIN (IGFBP) SERUM LEVELS AND HEPATIC IGFBP-2 AND -3 mRNA EXPRESSION IN DIABETIC AND INSULIN-TREATED SWINE (SUS SCROFA)*

MICHAEL E. WHITE,† DOUGLAS W. LEAMAN,‡ TIMOTHY G. RAMSAY,§ KIMBERLY A. KAMPMAN,‖ CATHERINE W. ERNST† and JEANNE M. OSBORNE‡

Department of Animal Science, University of Minnesota, St Paul, MN 55108, U.S.A. (Tel. 612 624-5370; Fax 612 625-5789); †Department of Animal Science, The Ohio State University, Columbus, OH 43210, U.S.A.; and §USDA, Beltsville, MD, U.S.A.

(Received 15 February 1993; accepted 19 March 1993)

Abstract—1. Diabetes had no significant effect on IGFBP-3 message and serum levels however, subsequent insulin treatment caused more than a two-fold increase in both hepatic IGFBP-3 mRNA and serum levels above controls (P < 0.05).
2. The induction of diabetes in pigs significantly increased the steady state levels of IGFBP-2 mRNA in the liver of young swine (P < 0.05).
3. Both liver message and serum IGFBP-2 were reduced to control levels with insulin therapy.
4. We report here that in addition to its affects on IGFBP-2, insulin is involved in the regulation of IGFBP-3 expression.

INTRODUCTION

Uncontrolled insulin-dependent diabetes mellitus (IDDM) results in growth retardation in humans, rats, pigs and other species. The insulin-like growth factors (IGFs) have been implicated as potentially important regulatory factors in IDDM. Serum IGF-I levels are consistently decreased with diabetes, and return to near normal levels following insulin therapy (Leaman et al., 1990; Fagln et al., 1989).

In biological fluids, IGFs are complexed to specific binding proteins which can modulate their biological action (Elgin et al., 1987; Clemmons et al., 1987; De Vroede et al., 1986; De Mellow and Baxter, 1988; Cornell et al., 1987; Gopinath et al., 1989; Walton et al., 1989; Blum et al., 1989). Postnatally, the majority of the circulating IGF is bound to IGFBP-3 in the 150 kDa IGF binding complex (Baxter, 1991). Presently the cDNAs for six different IGFBPs (IGFBP-1 through 6) have been cloned and sequenced (Shimasaki et al., 1991a,b). Two of the predominant IGFBPs found in porcine serum are IGFBP-3 and -2 (Coleman et al., 1991). Circulating levels of these two IGFBPs have been shown to be differentially regulated by growth hormone and nutritional status (Busby et al., 1988; McCusker et al., 1989; Clemmons et al., 1989). However, little is known about the hormonal and molecular regulation of these IGFBPs.

In this study we have utilized the pig to investigate the circulating IGFBPs and hepatic IGFBP-2 and IGFBP-3 mRNA expression in IDDM. This study provides new information dealing with the regulation of circulating and hepatic mRNA expression of the IGFBPs in swine. We demonstrate marked increases in circulating IGFBP-3 and liver IGFBP-3 mRNA with insulin treatment. In addition, our data reveal a marked increase in IGFBP-2 mRNA after the induction of diabetes which returns to control levels after insulin therapy in swine.

MATERIALS AND METHODS

Animals

Eighteen crossbred barrows (40 kg) were fitted with indwelling jugular catheters under anesthesia. Animals were housed in individual crates with free access to feed and water in a controlled environment, with timed lighting providing a constant 11 hr light and 13 hr dark cycle. Blood samples were collected at three hour intervals for 12 hr during the day. Diabetes was induced in 12 animals with IV streptozotocin injections (120 mg/kg) and diabetes was confirmed by the presence of hyperglycemia, glycosuria and hypoinsulinemia. Control animals (N = 4) were injected with vehicle. Insulin-treated animals (N = 5) were subcutaneously injected with 1 U/kg of a 1:1 mixture of porcine Iletin II Lente and Iletin II.

*Published as article No. 20,061 of the Scientific Journal Series of the Minnesota Agricultural Experiment station, Project No. 16-068. Supported in part by USDA Grant No. 87-37265-2598.
†To whom correspondence should be addressed at: Department of Animal Science, University of Minnesota, 137 Peters Hall, 1404 Gortner Ave., St. Paul, MN 55108, U.S.A.
Protamine Zinc insulins (Eli Lilly, Indianapolis, IN) twice daily for one week in order to normalize blood glucose levels. Fasted control (N = 2) and fasted-diabetic animals (N = 3) were maintained without feed for 3 days prior to slaughter. At the end of the 3 week study liver tissues were collected, immediately frozen in liquid nitrogen and stored at -70°C. All animal protocols were conducted following guidelines established by the National Institutes of Health. Animal use protocols were approved by the Animal Care and Use Committee.

Blood glucose

All serum samples were analyzed for blood glucose using a colorimetric, enzymatic, glucose oxidase reaction kit from Sigma (St Louis, MO).

RNA isolation and northern analysis

Total RNA was isolated from liver segments pooled (w/w) from animals in each treatment group by homogenization in 4 M guanidine thiocyanate followed by centrifugation through a cushion of 5.7 M CsCl for 22 hr at 20°C. After phenol/chloroform extractions and concentration determination, 50 μg of total liver RNA were denatured and electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde. Gels were then stained with ethidium bromide to insure equivalent loading and integrity of RNA and destained for 2 hr in distilled water. Total RNA was transferred for 18 hr by capillary action onto nitrocellulose membranes in 20 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate). After transfer, the membrane was rinsed briefly in 6 × SSC, air-dried for 30 min and vacuum-dried at 80°C for 1 hr. Gels were restained with ethidium bromide to verify that the transfer of RNA was complete.

Probes

The cDNA probe for rat IGFBP-2 were generous gifts from Dr Matthew Rechler (National Institutes of Health, Bethesda, MD). The cDNA probe for porcine IGFBP-3 was kindly provided by Dr Shunichi Shimasaki (The Whittier Institute for Diabetes and Endocrinology, La Jolla, CA). Probes were radiolabeled to a specific activity of 2 × 10⁶ cpm/μg cDNA using a random hexanucleotide priming kit from Amersham (Arlington Heights, IL). Prior to hybridization, nitrocellulose membranes were prehybridized for 2 hr at 65°C in rapid hybridization buffer purchased from Amersham (Arlington Heights, IL). Membranes were probed with 1.7 × 10⁶ cpm per blot, hybridized at 65°C for 16–18 hr, rinsed, using a standard protocols (Sambrook et al., 1989), and subjected to autoradiography. Autoradiographs were analyzed by video densitometry.

Radioimmunoassays

Serum IGF-1 levels were determined using a heterogeneous RIA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) following acid ethanol extraction as previously reported (Leaman et al., 1990). Various dilutions of acid ethanol-extracted swine serum exhibited parallelism with human IGF-1 standards. Inter-assay coefficient of variation was 14.5% and intra-assay was 2.5%. Insulin levels were measured utilizing an insulin radioimmunoassay (RIA) (Cambridge Medical Diagnostic, Billerica, MA). Insulin RIA results are expressed in uU/ml where 1 U is equivalent to 0.001 ng porcine insulin.

Ligand blotting

Five specific IGF binding protein bands were measured in serum from individual animals, using non-reducing sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) according to the procedure of Laemmli (1970) followed by ligand blotting with [125I]IGF-1. PAGE-separated protein bands were electrophoretically transferred on to nitrocellulose paper. After transfer to nitrocellulose, the blots were rinsed, quenched with 1% non-fat dry milk and then incubated with [125I]IGF-1 for 16 hr at 4°C with or without 100 ng/ml unlabeled IGF-1. The membranes were rinsed, dried and placed in a cassette with Kodak XAR film at -70°C and exposed for 7–10 days. All bands showing IGF binding were completely removed by incubation with unlabeled IGF-1. Video densitometry measurements of the autoradiograph provide quantitation of IGF binding proteins. Mᵣ were estimated by comparison to prestained protein standards. The standards used were: myosin heavy chain (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

Statistical analysis

Data were analyzed by analysis of variance using the General Linear Model procedure of PC SAS™ (1989) Release 6.04. In cases where main effects were significant, differences between means for preplanned comparisons were tested using Fisher’s least significant difference (LSD) test.

RESULTS AND DISCUSSION

In this study, we measured the hepatic expression of IGFBP-2 and IGFBP-3 under normal conditions and in diabetic swine subjected to insulin therapy or fasting. The results reported here represent new information on the effects of experimental IDDM and insulin treatment on circulating IGFBPs and hepatic IGFBP-2 and -3 mRNA expression in swine.

The induction of IDDM in swine, followed by subsequent insulin treatment, significantly affected serum parameters including blood glucose, serum insulin and serum IGF-1 levels as reported previously (Leaman et al., 1990). Streptozotocin treatment induced severe IDDM as indicated by a four-fold...
increase in blood glucose and non-detectable insulin levels. Serum IGF-I levels were decreased 74% with diabetes. IGF-I levels and blood glucose were restored to control levels with insulin therapy (Table 1).

Ligand blot analysis of serum IGFBPs from control, diabetic and insulin-treated animals (Fig. 1) revealed five IGFBP bands as has been previously reported for swine (White et al., 1991; McCusker et al., 1989; Coleman et al., 1991). The 43 and 39 M\(_{r}\) \(\times 10^{-3}\) bands are IGFBP-3 while the 32 M\(_{r}\) \(\times 10^{-3}\) band is IGFBP-2 (Coleman et al., 1991). Serum from insulin-treated diabetic pigs exhibited increased circulating IGFBP-3 levels over both non-diabetic controls and untreated diabetic pigs, whereas the 32 and 29 M\(_{r}\) \(\times 10^{-3}\) IGFBP bands in serum were significantly increased with diabetes and returned to control levels with insulin treatment.

Hepatic IGFBP-3 mRNA levels (Fig. 3) paralleled serum IGFBP-3 levels. Diabetes non-significantly depressed IGFBP-3 message and serum levels; however, subsequent insulin treatment caused more than a two-fold increase in both hepatic IGFBP-3 mRNA and serum levels above controls. The dramatic increase in serum IGFBP-3 and hepatic message with insulin treatment occurred concomitantly with the restoration of serum IGF-I levels. IGF-I has been shown to increase the production of IGFBP-3 in specific cell types (Cohick et al., 1992; Camacho-Hubner et al., 1991). Thus the increased IGFBP-3 expression may be caused by the increased circulating IGF-I. However, this does not appear to be a likely mechanism. The IGF-I levels following insulin treatment were not different from those of control animals whereas the IGFBP-3 serum and hepatic mRNA levels were greatly increased over controls. In addition, IGFBP-3 expression was affected very little by the sharp fall in circulating IGF-I levels observed in diabetic animals. Thus it does not seem likely that the restoration of serum IGF-I to normal levels should be the primary cause of the two- to three-fold increase in IGFBP-3 expression observed with insulin treatment in diabetic swine.

Many studies have demonstrated the dependence of IGF-I and IGFBP-3 serum levels and hepatic mRNA on growth hormone (GH) status under normal conditions. However, GH is not effective at restoring serum IGF-I levels in diabetic rats whereas insulin treatment does restore IGF-I levels in both rats and swine (Leaman et al., 1990; Fagin et al., 1989). In this study we demonstrate that diabetes has little, if any, effect on IGFBP-3 expression and that insulin treatment of diabetic animals significantly increases IGFBP-3 serum and hepatic mRNA levels. This suggests that in addition to GH, insulin is also involved in the regulation of IGFBP-3 serum levels and mRNA expression.

The induction of diabetes in pigs significantly increased the steady-state levels of IGFBP-2 mRNA in the liver of young swine (Fig. 2). This occurred in parallel with increased serum IGFBP-2 levels. Both liver message and serum IGFBP-2 were reduced to control levels with insulin therapy. Similar results have been reported in streptozotocin-diabetic rats (Ooi et al., 1990; Böni-Schnetzler et al., 1989; Rechlcr et al., 1991); however, insulin treatment did not reduce IGFBP-2 mRNA to normal in severely diabetic–ketotic rats whereas it did in non-ketotic diabetic rats induced with lower levels of streptozotocin (Böni-Schnetzler et al., 1989). The effects of insulin treatment on hepatic IGFBP-2 mRNA in diabetic swine reported here are consistent with observations using cultured rat hepatocytes indicating that insulin is primarily responsible for decreasing IGFBP-2 mRNA levels. Serum ligand blots indicated that diabetic swine subjected to a 3-day fast exhibited increased serum IGFBP-2 (32 M\(_{r}\) \(\times 10^{-3}\) band) levels (Fig. 1). However, no corresponding increase in IGFBP-2 mRNA was observed. This bears further investigation since, in rats, fasting increases hepatic IGFBP-2 mRNA levels (Ooi et al., 1990; Orlowski et al., 1990; Rechlcr et al., 1991).

This study employed streptozotocin-induced diabetes in young swine to investigate the regulation of serum IGFBPs and hepatic IGFBP-2 and IGFBP-3 mRNA expression. The induction of diabetes had little effect on IGFBP-3 serum or hepatic mRNA levels whereas it sharply increased IGFBP-2 levels and hepatic mRNA expression. Insulin treatment of diabetic swine caused a three-fold increase in serum IGFBP-3 levels and liver mRNA expression while it reduced IGFBP-2 expression down to control levels. These results provide new information regarding serum IGFBP levels and the hepatic mRNA expression of IGFBP-2 and IGFBP-3 with diabetes and insulin therapy in swine. We report here that, in addition to its regulatory affects on IGFBP-2, insulin

### Table 1. Blood glucose, serum insulin and serum IGF-I levels in streptozotocin diabetic swine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood glucose (mg/dl)</th>
<th>Serum insulin (uU/ml)</th>
<th>Serum IGF-I (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>112.5 ± 3.5(^a)</td>
<td>11.2 ± 1.44(^b)</td>
<td>115.2 ± 5.2(^b)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>509.0 ± 26.4(^b)</td>
<td>*</td>
<td>30.2 ± 7.1(^b)</td>
</tr>
<tr>
<td>Diabetic insulin</td>
<td>102.0 ± 24.1(^b)</td>
<td>30.2 ± 1.41(^b)</td>
<td>126.8 ± 2.8(^b)</td>
</tr>
<tr>
<td>Diabetic fasted</td>
<td>140.0 ± 55.2(^b)</td>
<td>*</td>
<td>14.3 ± 2.1(^b)</td>
</tr>
<tr>
<td>Control fasted</td>
<td>82.5 ± 3.5(^b)</td>
<td>2.85 ± 0.21(^b)</td>
<td>24.7 ± 1.2(^b)</td>
</tr>
</tbody>
</table>

Mean ± SE.

\(^{a,b}\)Values within a column with different superscripts differ (P < 0.01).

*Below detectable levels.
Fig. 1. A. Ligand blot. Representative autoradiograph of a ligand blot containing 2 µl sera from control (C), diabetic (D), diabetic-insulin-treated (DI), fasted-diabetic (DF) and fasted-control (CF) animals. Binding proteins were separated on a 7–15% polyacrylamide gradient gel under non-reducing conditions using SDS–PAGE, transferred electrophoretically to nitrocellulose and probed with [125I]IGF-1. B. Ligand blot signal intensity. Video densitometry of IGFBP bands (43 and 39, 32, 29 and 24 Mr × 10⁻³ bands) from ligand blot autoradiographs of individual serum samples from animals in each treatment group. Means ± SE of relative signal intensity for each IGFBP band are shown for each treatment. Means with different letters are significantly different between ages (P < 0.05).
Fig. 2. A. Northern blot of liver IGFBP-2 mRNA. Representative autoradiograph of northern blot analysis of total liver RNA from control (C), diabetic (D), diabetic-insulin-treated (DI), fasted-diabetic (DF) and fasted-control (CF) animals. Total RNA was fractionated on a 2.2 M formaldehyde, 1% agarose gel, transferred to nitrocellulose and hybridized with a 32P labeled rat IGFBP-2 cDNA. Photograph of ethidium-bromide stained gel prior to transfer demonstrates consistency of loading and integrity of rRNA in the samples. B. Signal intensity of liver IGFBP-2 mRNA. Hybridization signal of Northern blots of total liver RNA probed with a rat cDNA for IGFBP-2 quantified by video densitometry. Solid bars represent mean densitometry measurements + SE for animals from each treatment group. Means with different letters are significantly different (P < 0.05).
Fig. 3. A. Northern blot of liver IGFBP-3 mRNA. Representative autoradiograph of northern blot analysis of total liver RNA from control (C), diabetic (D), diabetic-insulin-treated (DI), fasted-diabetic (DF) and fasted-control (CF) animals. Total RNA was fractionated on a 2.2 M formaldehyde, 1% agarose gel, transferred to nitrocellulose and hybridized with a 32P labeled porcine IGFBP-3 cDNA. Photograph of ethidium-bromide stained gel prior to transfer demonstrates consistency of loading and integrity of rRNA in the samples. B. Signal intensity of liver IGFBP-3 mRNA. Hybridization signal of northern blots of total liver RNA probed with a porcine cDNA for IGFBP-3 quantified by video densitometry. Solid bars represent mean densitometry measurements ± SE for animals from each treatment group. Means with different letters are significantly different (P < 0.05).
is also involved in the regulation of IGFBP-3 expression.

Acknowledgements—The authors would like to thank Jodie Duffy, Lielal De Sevilla and Rong Diao for their assistance in the study, and Dr William Dayton and Dr Marcia Hathaway for their assistance with the manuscript. We also thank Dr Matthew Rechler for providing the IGFBP-2 cDNA and Dr Shunichi Shimasaki for providing the IGFBP-3 cDNA.

REFERENCES


