Relation of Rat Fetal Body Weight to the Relative Expression of Insulin Genes I and II

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This study investigates the relation between the ratio of the abundance of mRNAs encoded by insulin genes I and II (insulin I/II mRNA ratio) and the weight of rat fetuses on gestational day 20. Total RNA was extracted from the pancreas of the fetuses with the maximum and minimum body weight in each litter on gestational day 20. The amount of insulin mRNAs I and II in each RNA preparation was determined by reverse transcription–polymerase chain reaction (RT-PCR) analysis and restriction enzyme digestion. The maximum and minimum weight of the fetuses were 4.07 ± 0.23 and 3.23 ± 0.34 g, respectively (N = 18, $P < .01$) and the corresponding insulin I/II mRNA ratios were 3.65 ± 0.55 and 1.42 ± 0.21 (N = 18, $P < .01$). Furthermore, the insulin I/II mRNA ratio correlated significantly with fetal weight ($r = .526$, $P < .05$). These results suggest that the relative expression of insulin genes I and II may affect the extent of fetal growth.

INSULIN is an important regulator of growth and metabolism in vertebrates. It is considered a fetal growth factor, because hyperinsulinemia in the fetus is associated with macrosomia and insulin deficiency with poor fetal development in humans and other species. Rats,[1,2] mice,[3] and certain fish[4] express two types of insulin, which were first identified in the rat pancreas by ion-exchange chromatography.[5] The mRNAs produced from the two rat nonallelic insulin genes are 92% identical in the coding regions, differing by only 34 of 439 nucleotides.[6,7] Rat preproinsulin I and II, the initial translation products, differ by three amino acids in the preregion, two in the B chain, and two in the C peptide.[6-10]

Freshly isolated islets of Langerhans from normal rats contain 58% insulin I and 42% insulin II,[11] and rat insulin I and II are synthesized at a ratio of 60:40.[1,12-14] Although the two rat insulin genes appear to function independently,[15] little is known about the regulation of their expression.

We previously showed that the ratio of insulin I mRNA to insulin II mRNA (insulin I/II mRNA ratio) in the fetal rat pancreas peaks at the time of birth, after which it decreases to the value characteristic of adult animals.[16] No such peak was apparent in fetuses from diabetic mothers, suggesting that the expression of insulin genes I and II is regulated differentially in the pancreas of fetal rats around the time of delivery.[17]

To investigate the possible relation between the expression of insulin genes I and II and fetal weight, we have now determined the insulin I/II mRNA ratio in the pancreas of fetuses with the maximum and minimum weight within litters on gestational day 20.

MATERIALS AND METHODS

Animals

Virgin female Sprague-Dawley rats (Shizuoka, Hamamatsu, Japan) with a body weight of 150 to 250 g were caged overnight with a male. The day of sperm-plug positivity was designated as gestational day 0. Eighteen maternal rats were used in these experiments. On gestational day 20, the fetuses were rapidly removed from the uteri of the pregnant rats after anesthesia by intraperitoneal injection of sodium pentobarbital (50 to 150 mg/kg body weight). The fetuses were quickly weighed, and the pancreas of the fetuses with the maximum and minimum weight in the same litter (18 fetuses in each group) was removed under a dissecting microscope, frozen immediately in liquid nitrogen, and stored at −80°C until analysis.

RNA Preparation

Total RNA was prepared from the pancreas by the acid guanidinium thiocyanate-phenol-chloroform method.[18] The tissue was homogenized with 500 μL of a solution containing 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate (pH 7.0), 0.5% sodium sarcosyl, and 0.1 mol/L 2-mercaptoethanol. After the addition of 50 μL 2-mol/L sodium acetate (pH 4.0), 500 μL phenol (saturated with water), and 100 μL chloroform, the homogenate was mixed thoroughly by inversion, shaken vigorously for 10 seconds, cooled on ice for 15 minutes, and centrifuged at 10,000× *g* for 20 minutes at 4°C. The aqueous phase was transferred to a new tube, mixed with 400 μL isopropanol, and kept at −80°C for 1 hour to precipitate the RNA. After centrifugation at 10,000× *g* for 20 minutes, the resulting RNA pellet was dried under a vacuum, treated with RNase-free DNase, and quantified by measuring the absorbance at 260 nm. The integrity of the RNA was assessed by agarose gel electrophoresis and visualization of 18S and 28S ribosomal RNA by ethidium bromide staining.

Reverse Transcription–Polymerase Chain Reaction Analysis

The reverse transcription (RT) reaction was performed for 60 minutes at 37°C in a 50-μL mixture containing 10 μg total RNA, 1× RT buffer (GIBCO BRL, Gaithersburg, MD), 0.1 mol/L dithiothreitol, 10 mmol/L of each deoxynucleoside triphosphate, 40 U RNase inhibitor, 10 pmol of a 19-nucleotide (nt) antisense primer, and 200 U Moloney murine leukemia virus (GIBCO BRL). The resulting cDNAs were amplified with a 21-nt sense primer (primer 1, 5′-ATTGTTCCAACATGGCCCTGT, nt 47 to 67) and a 19-nt antisense primer (primer 2, 5′-AACTGGAGAACTACTGCAA, nt 368 to 386) that yield polymerase chain reaction (PCR) products of 340 base pairs (bp) for both insulin cDNAs I and II. The PCR was performed in a 50-μL mixture containing Taq polymerase buffer, 10 mmol/L of each deoxynucleoside triphosphate, 2.5 IU Taq DNA polymerase (Pharmacia, Uppsala, Sweden), 10 pmol sense primer, 10 pmol [α-^{32}P]ATP-labeled antisense primer, and cDNA. Amplification was achieved by 30 cycles of incubation for 1 minute at 94°C, 2 minutes at 57°C, and 1 minute at 72°C. A 10-μL

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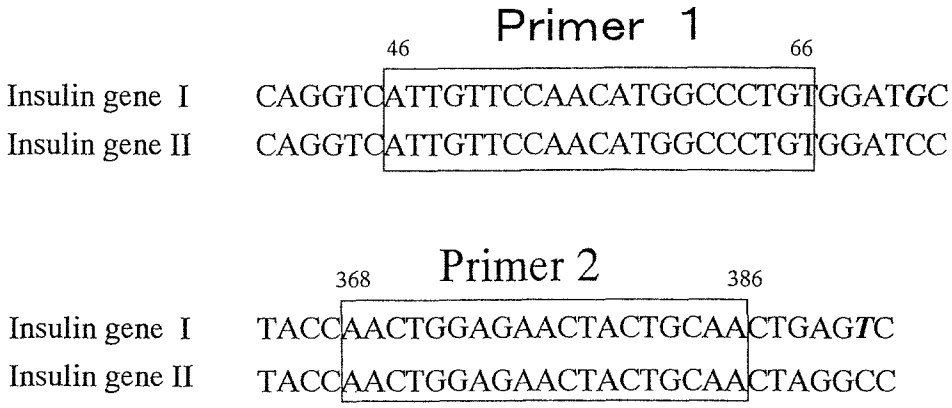

Fig 1. Rat insulin I primers amplified rat insulin I and rat insulin II primers amplified rat insulin II, indicating the specificity of the primer.

portion of each reaction mixture was subjected to electrophoresis in a 5% polyacrylamide gel containing ethidium bromide (0.5 μg/mL). The gel was then photographed under UV light.

Restriction Enzyme Analysis of PCR Products

The RT-PCR products of rat insulin mRNAs I and II were digested with the restriction enzymes *Rsa*I or *Sma*I, and the resulting fragments were resolved on a 5% polyacrylamide gel containing ethidium bromide. The PCR product of the insulin I cDNA is cleaved by *Rsa*I at nt 175 to yield fragments of 129 and 211 bp, whereas the product of the insulin II cDNA is cleaved by *Sma*I at nt 272 to yield fragments of 226 and 114 bp. The gels were cut into 1.5-mm slices, and the radioactivity in each slice was determined by scintillation spectroscopy.

Sequencing of PCR Products

PCR products were ligated into the pCRII vector (Invitrogen, San Diego, CA), which was then used to transfect *Escherichia coli*. White colonies were selected and grown, and after the insert length was confirmed, the cloned product was isolated from the plasmid, purified by column chromatography, and sequenced by the double-stranded dideoxy method.

Statistical Analysis

Data are presented as the mean ± SE and were analyzed by Student's *t* test. A *P* value less than .05 was considered statistically significant. Simple linear regression analyses were performed to evaluate the associations between fetal weight and the insulin I/II mRNA ratio.

RESULTS

Single-stranded cDNA was generated by RT with primer 2 from pancreatic total RNA isolated from a fetus of a normal mother on gestational day 20. The cDNA was then amplified by PCR with primers 1 and 2 (Fig 1). No PCR products were generated from the RNA sample without prior RT. The sequences of the PCR products derived from insulin mRNAs I and II differed at only 24 of 340 nt. Exposure of the PCR products to *Rsa*I resulted in digestion of the insulin I product to fragments of 129 and 211 bp, whereas *Sma*I cleaved the insulin II product into fragments of 226 and 114 bp (Figs 2 and 3). The example data are shown in Fig 4.

The amplification efficacy of insulin cDNAs I and II by PCR was assessed by subjecting the corresponding plasmids in a ratio of 5:1, 3:2, 1:1, 2:3, or 1:5 to the PCR protocol and analyzing the resulting products by restriction enzyme digestion. The relative radioactivity associated with the PCR products of insulin genes I and II corresponded to the initial ratio of the plasmids.

The maximum and minimum weight of fetuses within a litter on gestational day 20 was 4.07 ± 0.23 and 3.23 ± 0.34 g, respectively (N = 18, $P < .01$). The insulin I/II mRNA ratio was 3.65 ± 0.55 and 1.42 ± 0.21 for the fetuses with maximal and minimal body weight, respectively (N = 18, $P < .01$). The insulin I/II mRNA ratio showed a significant correlation with fetal weight (Fig 5).

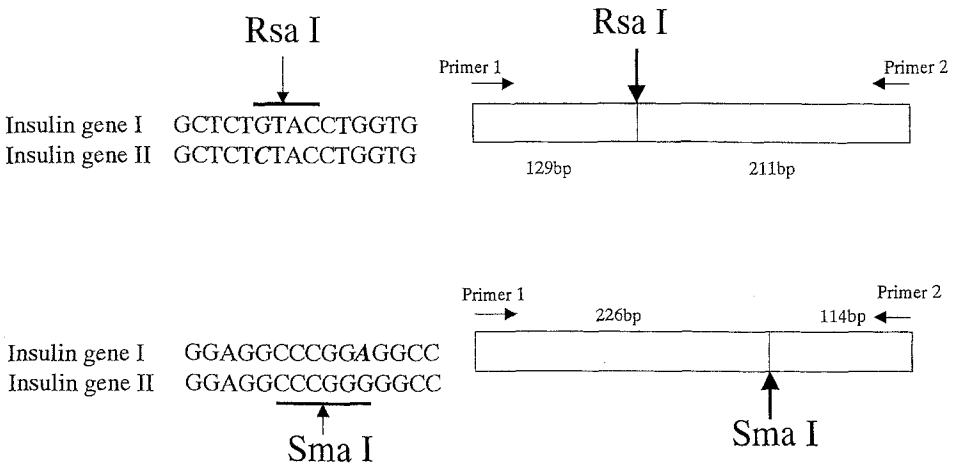

Fig 2. Restriction enzyme analysis of PCR product obtained from the pancreas at gestational day 20. The size of the resulting insulin gene fragments was determined by digestion with *Rsa*I and *Sma*I.

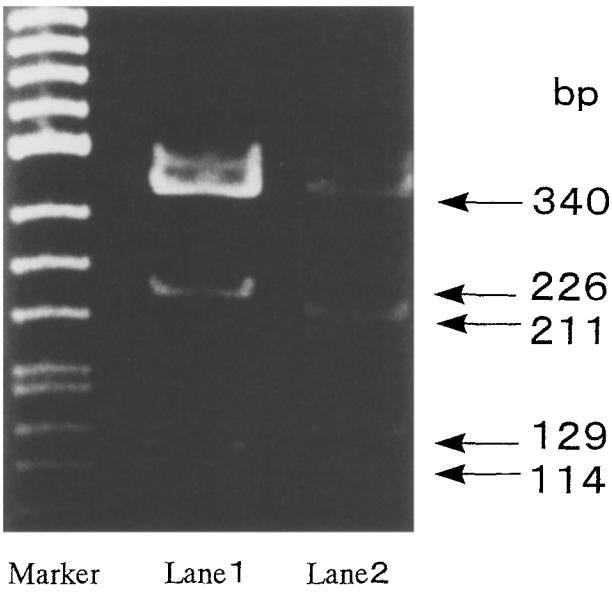

Fig 3. Restriction enzyme analysis of PCR products obtained from the pancreas of a fetus from a normal mother on gestational day 20. PCR products were digested with *Sma*I (lane 1) or *Rsa*I (lane 2), and the resulting fragments were separated on a 5% polyacrylamide gel. Marker, molecular size standard (*Hin*fI digest of .X174DNA).

DISCUSSION

Insulin has an important trophic role in fetal and neonatal development, and its availability during this period is maintained by ongoing islet cell proliferation. During the final 2 days of gestation, the body weight of the rat fetus approximately doubles, while the weight of the pancreas increases 5-fold. Insulin-like growth factors (IGFs) I and II, as well as IGF-binding proteins, have been implicated as paracrine regulators of islet cell growth within the developing pancreas.[19-21]

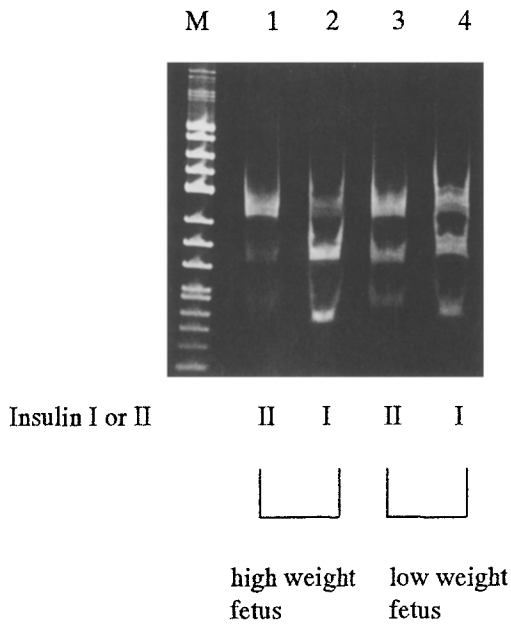

Fig 4. Example of the data in the current study. In these data, 2 fetuses, ie, high- and low-weight fetuses, were used as an example. Lanes 1 and 3, results from insulin II; lanes 2 and 4, results from insulin I.

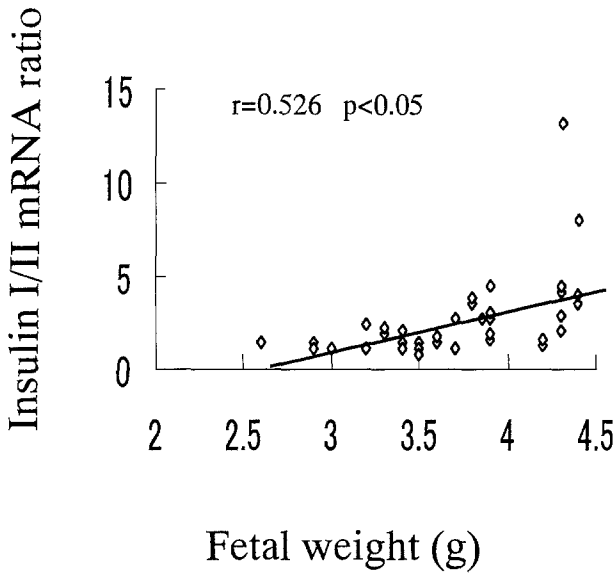

Fig 5. Correlation between the insulin I/II mRNA ratio and fetal weight.

The precise role of insulin during fetal development is not known. Although it does not cross the fetoplacental barrier, insulin may be important for maintaining and promoting fetal growth even before pancreatic differentiation. Glucose is the main physiological regulator of insulin biosynthesis and secretion, and the magnitude of its effect is proportional to its rate of intracellular metabolism. Insulin biosynthesis increases 5- to 10-fold within minutes of islet exposure to high glucose in vitro, and the increase persists as long as glucose remains elevated. The glucose-induced increase in insulin biosynthesis is mediated at both the transcriptional and posttranscriptional levels.

Fetuses and neonates of manifestly diabetic rats contain a reduced β-cell mass.[22] In addition, these animals show both a reduced pancreatic insulin content[23] and a slower rate of insulin biosynthesis.[24] These observations have been interpreted to indicate delayed growth and development of the endocrine pancreas in the offspring of diabetic rat mothers.[22] Yoshinari and Daikoku[25] showed that insulin appears in the pancreas of normal rat fetuses on day 12.5 of fetal development, the cell arrangement shows a mature pattern on day 18.5, and islets are functionally mature on day 18. Eriksson and Swenne[22] observed that the actual weight of the β cell increases 2-fold during the last 2 days of the gestational period. Watts et al[26] showed that the weight of the pancreas increases 15-fold, the percentage of insulin-containing β cells increases 12-fold, and insulin accumulation in the pancreas increases about 200-fold during the last 5 days of the gestational period. Thus, the fetal pancreas undergoes marked development in the late gestational period; however, its ability to secrete insulin in response to a rapid glucose challenge is less well developed such that the insulin concentration in fetal blood is unrelated to the glucose concentration. Fetal insulin is therefore considered to function as a fetal growth factor, as well as a regulator of the blood glucose concentration.

The rat insulin gene II corresponds to the single insulin gene of other vertebrates. However, it is not clear whether the translational mechanism of regulation of rat insulin II expression previously characterized[27] also applies to the control of insulin expression in other vertebrates.

During the course of normal gestation in the rat, circulating insulin in the fetus as measured by radioimmunoassay achieves maximal concentrations over the last 3 days of gestation and then decreases markedly immediately after birth.[26,28] In the developing rat pancreas, preproinsulin mRNA is first observed on day 15 of gestation and then increases rapidly until day 2 after birth.[29] The accumulation of insulin in pancreatic β cells is proportional to the accumulation of proinsulin mRNA, suggesting that insulin synthesis during pancreatic development is controlled predominantly by transcription of the insulin genes. The developmental expression of rat insulin genes is probably not activated by glucose, because the blood glucose concentration in the rat fetus is low and does not increase until after birth. Whether insulin expression during normal fetal development is also controlled at the level of translation has not been determined. However, given that translational control of insulin biosynthesis occurs only at relatively high glucose concentrations, the transcriptional control of insulin expression likely predominates during fetal development.

We previously demonstrated an effect of the maternal environment on insulin gene expression in the fetal rat pancreas at the time of delivery.[16] In the pancreas of fetuses from normal mothers, an increase in insulin gene I expression resulted in a maximal insulin I/II mRNA ratio at the time of delivery, with the ratio rapidly achieving adult levels thereafter. In contrast, in the pancreas of fetuses from diabetic mothers, no increase in insulin gene I expression was apparent, so the insulin I/II mRNA ratio at the time of delivery was similar to that of adult rats.[17] In the present study, the maternal environment was the same for fetuses with the highest and lowest body weight. We showed that the insulin I/II mRNA ratio in the pancreas was significantly greater for the fetuses with the maximal body weight than for those with the minimal body weight.

Together with the fact that fetuses of diabetic mothers show hypoplasia and no increase in insulin gene I expression at the time of delivery, unlike the fetuses of normal mothers, the present data suggest that the role of insulin I as a growth factor during fetal development may be more important than that of insulin II. Alternatively, the interaction of insulins I and II may be important for their trophic effects, so that an imbalance in their relative concentration may have a deleterious effect on fetal growth. Finally, it is also possible that an increase in fetal body weight may be the cause rather than the consequence of an increase in the insulin I/II mRNA ratio.

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