

Altered Nephrogenesis Due to Maternal Diabetes Is Associated With Increased Expression of IGF-II/Mannose-6-Phosphate Receptor in the Fetal Kidney

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We have recently demonstrated that the exposure to hyperglycemia in utero impairs nephrogenesis in rat fetuses (Amri K et al., *Diabetes* 48:2240–2245, 1999). Diabetic pregnancy is commonly associated with alterations in the IGF system in fetal tissues. It has also been shown that both IGF-I and IGF-II are produced within developing metanephros and promote renal organogenesis. Therefore, we investigated the effect of maternal diabetes on IGFs and their receptors in developing fetal rat kidney. Diabetes was induced in pregnant rats by a single injection of streptozotocin on day 0 of gestation. We measured the amounts of IGF and their receptors, both proteins and mRNAs, in the metanephroi of fetuses issued from diabetic subjects and in age-matched fetuses from control subjects (14–20 days of gestation). IGF-II was produced throughout fetal nephrogenesis, whereas IGF-I protein was not detected, suggesting a critical role of IGF-II in kidney development. Fetal exposure to maternal diabetes caused no change in IGF production in the early stages of nephrogenesis. Similarly, the amounts of IGF-I receptor and insulin receptor were not altered. By contrast, there was an increase in production of IGF-II/mannose-6-phosphate receptor throughout nephrogenesis. Because this receptor plays an essential role in regulating the action of IGF-II, the altered nephrogenesis in fetuses exposed to maternal diabetes may be linked to a decrease in IGF-II bioavailability. *Diabetes* 50:1069–1075, 2001

More than 12% of pregnancies in the U.S. occur in diabetic women. The resulting children are at higher-than-normal risk of having congenital malformations, making this a serious endocrine problem (1–4). These malformations result from defects occurring in early organogenesis (2,5), including failure of neural tube closure, caudal regression syndrome, and urogenital abnormalities, which can be as severe as renal agenesis (2,6–8). In a recent study (9), we demon-

strated that exposure to hyperglycemia in utero impairs nephrogenesis in the rat, leading to a reduced number of nephrons. An inborn nephron deficit, even a moderate one, is a risk factor for the development of chronic renal disease and hypertension in adulthood (10–13). The factors underlying these developmental abnormalities remain obscure.

Diabetic pregnancy is commonly associated with alterations in the expression or bioavailability of IGFs in several fetal organs (14–18). However, the impact of maternal diabetes on the expression of IGFs and their receptors in fetal kidney has not yet been examined.

IGF-I and IGF-II are produced within developing metanephros (19,20) and promote renal organogenesis (21,22). The signaling of IGF is mediated by IGF-I receptor (IGF-IR), which is a heterotetrameric transmembrane glycoprotein with tyrosine kinase activity that resembles the insulin receptor (IR) (23). Recent gene-targeting experiments have demonstrated that the IR mediates some of the growth-promoting functions of IGF-II during mouse embryogenesis (24). IGF-II also binds with high affinity to a type 2 receptor (IGF-II/mannose-6-phosphate receptor [M6PR]) that is devoid of tyrosine kinase activity and is identical to M6PR. IGF-II/M6PR is involved in the clearance of IGF-II by receptor-mediated endocytosis (25). Both receptors are present in developing rodent kidneys. In vitro studies in which growth of metanephroi was prevented by the addition of antibodies to IGFs or IGF-II/M6PR and by the addition of antisense oligodeoxynucleotides to IGF-IR suggest that these receptors are involved in renal development (19,20).

Therefore, we investigated the effect of maternal diabetes on IGFs and their receptors in developing fetal kidney. The expression of genes encoding IGF-I, IGF-II, IGF-IR, IGF-II/M6PR, and IR in metanephros issued from control fetuses and fetuses exposed to hyperglycemia during gestation was quantified by Northern and Western blotting. In addition, the intrarenal distribution of IGF-II/M6PR during the early stage of nephrogenesis was localized by *in situ* hybridization.

RESEARCH DESIGN AND METHODS

Animals. Female Sprague-Dawley rats, weighing 200–300 g and given free access to water and standard laboratory diet (UAR Laboratory, Villemoisson sur Orge, France), were caged overnight with male rats; vaginal smears were collected the following morning and tested for pregnancy. The day a positive result was obtained was designated as day 0 of gestation.

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Received for publication 8 August 2000 and accepted in revised form 16 January 2001.

IGF-IR, IGF-I receptor; IR, insulin receptor; M6PR, mannose-6-phosphate receptor; STZ, streptozotocin; TBST, Tris-buffered saline with Tween.

TABLE 1
Specific conditions used for electrophoresis and immunodetection in Western blot experiments

	Electrophoresis		Immunodetection		
	Reducing condition	Gradient	Host antibody	Dilution	Milk
IGF-I	—	10–30%	Rabbit	1/500	5%
IGF-II	—	10–30%	Mouse	1/500	5%
IGF-IR	β -mercaptoethanol (143 mmol/l)	6–10%	Rabbit	1/2,000	5%
IGF-II/M6PR	—	6–10%	Rabbit	1/10,000	10%
IR	β -mercaptoethanol (143 mmol/l)	6–10%	Rabbit	1/500	5%

Diabetes was induced in the pregnant female rats by a single injection of 40 mg/kg body wt of streptozotocin (STZ; Sigma, Saint Quentin Fallavier, France) on day 0 of gestation. STZ was diluted in 0.4 mol/l citrate buffer, pH 4.5. Control animals were given an equivalent amount of citrate buffer. Maternal blood samples were taken every 2 days from the cut tip of the tail, and the plasma glucose concentration was determined immediately by the glucose-oxidase technique using a glucose analyzer (Beckman Instruments, Fullerton, CA). The mean maternal plasma glucose concentration of the STZ group remained almost constant until the experimental day and was approximately three times higher (18.7 ± 0.1 mmol/l) than in the controls (5.27 ± 0.05 mmol/l, $n = 45$ for both groups).

Fetuses were removed from anesthetized pregnant female rats on days 14, 15, 16, 18, and 20 of gestation. Metanephroi were surgically removed from embryos, immediately frozen in liquid nitrogen, and stored at -80°C . For RNA and protein extraction, fetal kidneys were pooled from one to three litters at 14, 15, and 16 days of gestation and from two fetuses at 18 and 20 days of gestation.

Isolation of RNA and protein and probe preparation and labeling. RNA and proteins were isolated from the same sample by one-step liquid-phase separation using the Triagent procedure (Gibco BRL, Grand Island, NY). The precipitated RNA was resuspended in sterile H_2O and quantified by measuring the absorbance at 260 nm. The protein pellet was dissolved in 1% SDS. The protein content of the tissue extracts was determined using the Bio-Rad DC protein assay (Bio-Rad, Marne la Coquette, France). Dilutions of bovine serum albumin were used as protein standards.

The cDNA probes were labeled with [^{32}P]dCTP using Rediprime DNA labeling system (Amersham, Arlington, IL) and purified on NucTrap Probe Purification columns (Stratagene, Cambridge, U.K.).

Northern blot analysis. RNA samples (15 μg) were denatured, fractionated by electrophoresis, and then transferred onto nylon membranes as previously described (26). Northern blots were prehybridized and then hybridized with [^{32}P]dCTP-labeled cDNA probes. The blots were exposed to X-ray film (Reflection; DuPont NEN, Boston, MA) for 6 h and up to 7 days at -80°C using intensifying screens. The blots were stripped in between hybridizations with different probes by washing for 1 min at 80°C in $0.1\times$ sodium chloride–sodium citrate and 0.1% SDS. The consistency of the relative amounts of total RNA loaded into each lane was checked by probing the blots with [^{32}P]dCTP-labeled cDNA-encoding 18 S ribosomal RNA. Signal intensity was quantified by densitometric analysis of autoradiograms using image analysis software (NIH Image).

Western blot analysis. A protein extract (25 μg) was boiled for 5 min with SDS–dissociation buffer (0.125 mol/l Tris-HCl, pH 6.8, 4% SDS, 20% glycerol) with or without β -mercaptoethanol (Table 1). Proteins were separated by electrophoresis in a discontinuous SDS–polyacrylamide gel gradient. Proteins were transferred to nitrocellulose membranes (Hybond-C Extra; Amersham) and the membranes were blocked by incubation for 1 h with 5–10% dried skim milk in Tris-buffered saline with Tween (TBST; 50 mmol/l Tris-HCl, 150 mmol/l NaCl, 0.05% Tween 20, pH = 8). Specific proteins were detected by incubating with antibodies suitably diluted in TBST (Table 1) for 1 h at room temperature. The membranes were then washed three times in TBST, and the bound primary antibody was detected with a peroxidase-antiperoxidase system (Jackson ImmunoResearch Laboratories, West Grove, PA). Antigen-antibody complexes were detected by enhanced chemiluminescence, as recommended by the manufacturer (Amersham). Finally, each membrane was labeled with a monoclonal anti- β -actin antibody to normalize the amount of protein loaded. Bands were quantified by densitometry using image analysis software (NIH Image).

In situ hybridization. In situ hybridization was performed in kidney of fetuses on day 16 of gestation as previously described (27,28), using 4% paraformaldehyde-fixed, paraffin-embedded tissues and the ^{35}S -labeled IGF-II/M6PR probe. For controls, tissue sections were first treated with ribonuclease A for 30 min and then washed, permeated, and hybridized with the specific probe.

cDNA probes and antibodies. Monoclonal anti- β -actin antibody was obtained from Sigma (St. Quentin Fallavier, France). Anti-IGF-I and anti-IGF-IR β -subunit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-IGF-II antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-IGF-II/M6PR was a gift from Dr. MacDonald (University of Nebraska Medical Center, Lincoln, NE). Anti-IR β -subunit was obtained from Chemicon (Temecula, CA).

IGF-I and IGF-II cDNA probes were a gift from Professor Rotwein (University of Washington Medical Center, Seattle, WA). Dr. Haugel (Endocrinologie Métabolisme et Développement, CNRS UPR 1,524, Meudon, France) kindly provided the IGF-IR cDNA probe. The IGF-II/M6PR cDNA probe was kindly provided by Dr. A. Clément (INSERM U515, Hôpital Trousseau, Paris, France), and the IR cDNA probe was a gift from Dr. B. Maitre (Hôpital Henri Mondor, Créteil, France).

Statistics. Differences between developmental stages were calculated by analysis of variance. Control and hyperglycemic groups, at the same stage, were compared by Student's unpaired *t* test. The threshold of significance was $P < 0.05$. All values are expressed as means \pm SE.

RESULTS

IGFs in fetal kidney. IGF-I mRNA was detected as a 7.5-kb band by Northern blotting. Densitometric analysis in the control group revealed a constant expression between days 14 and 20 of gestation (Fig. 1). IGF-I protein was under detection limit by Western blotting. The amounts of IGF-I mRNA in the diabetic and control fetuses were the same (Fig. 1).

The IGF-II cDNA probe detected a single major 3.6-kb mRNA band. IGF-II mRNA was abundant on days 14 and 15 of gestation and then declined gradually until day 20 (Fig. 2A). IGF-II protein was found as a specific hybridizing band of ~ 12 kDa. Band-densitometric analysis revealed that IGF-II was abundant at the early stage of fetal development (days 14–16 of gestation) and then decreased by $\sim 50\%$ on day 20 of gestation (Fig. 2B). The amounts of IGF-II mRNA and protein in fetuses from diabetic subjects were unchanged during the first stages of fetal nephrogenesis and then increased on day 20 of gestation by ~ 60 and 80%, respectively, as compared with age-matched fetuses from control subjects (Fig. 2A and B).

IGF receptors and IR in fetal kidney. The IGF-IR cDNA probe detected a single 11-kb mRNA band. Western blot analysis demonstrated a specific labeled band at ~ 90 kDa corresponding to the β -subunit of this receptor. Densitometric analysis (Fig. 3A and B) showed that mRNA expression was constant throughout fetal development, whereas the protein expression increased sharply on day 20 of gestation, suggesting a posttranslational regulation. The amounts of IGF-IR mRNA and protein were not altered by maternal diabetes (Fig. 3A and B).

Specific bands of 7 and 9 kb were observed in Northern blot hybridization analysis for IR. The 97-kDa band specific for the β -subunit of the IR was detected by Western blot analysis. The amounts of mRNA and protein increased

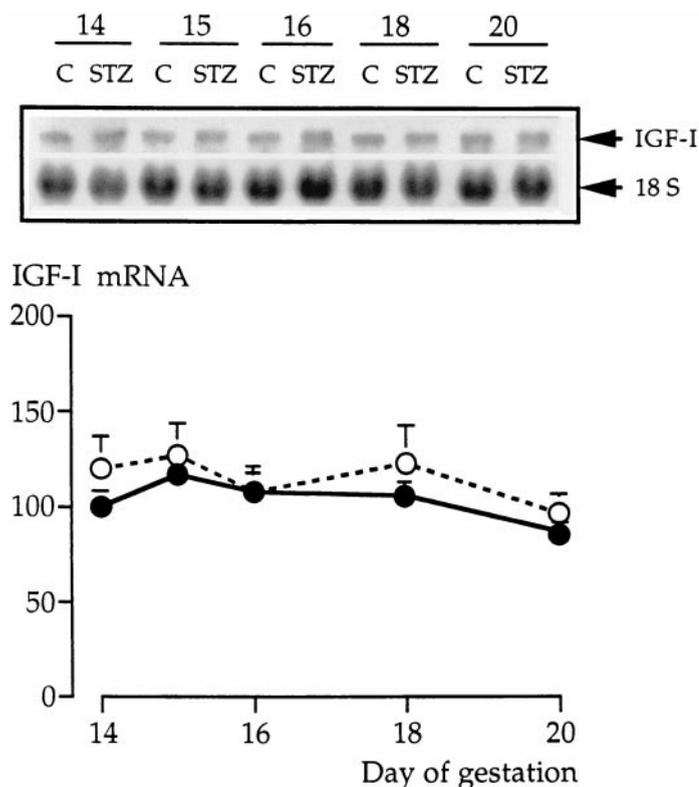


FIG. 1. Patterns of IGF-I mRNA in the kidneys of fetuses (14–20 days of gestation) from normal (plain line) and diabetic (broken line) rats. Values are means \pm SE of five to six experiments. Densitometric results are expressed as percentage of the control (day 14) value after normalization for RNA loading on the basis of hybridization of 18 S rRNA.

on day 18 of gestation. Then, between days 18 and 20, mRNA expression remained constant, whereas protein expression increased (Fig. 4A and B). Maternal diabetes did not alter the amounts of IGF-IR mRNA and protein (Fig. 4A and B).

The 9.5-kb IGF-II/M6PR mRNA was abundant in controls on day 14 of gestation and then decreased by \sim 50% on day 20 of gestation. Western blot analysis showed a specific band at \sim 250 kDa that had the same expression profile as the mRNA (Fig. 5A and B). However, the expression of IGF-II/M6PR mRNA was increased from day 15 of gestation in fetuses from diabetic subjects. IGF-II/M6PR protein also increased throughout the study period (Fig. 5). The greatest increase was on day 20 of gestation (75% for mRNA and 84% for protein as compared with age-matched control fetuses).

In situ hybridization in metanephros on day 16 of gestation showed that IGF-II/M6PR mRNA was localized in the epithelium, the ureteric bud, and in the neighboring domain of committed metanephric mesenchyme, which will differentiate into nephrons. It was also localized in the nonmature glomeruli, the S-shaped bodies (Fig. 6).

DISCUSSION

We have previously shown that exposure to hyperglycemia in utero leads to a nephron deficit (9). The mechanisms whereby hyperglycemia alters nephrogenesis are unknown. The major finding in this study is an increase in the amount of IGF-II/M6PR in the kidneys of fetuses from diabetic subjects.

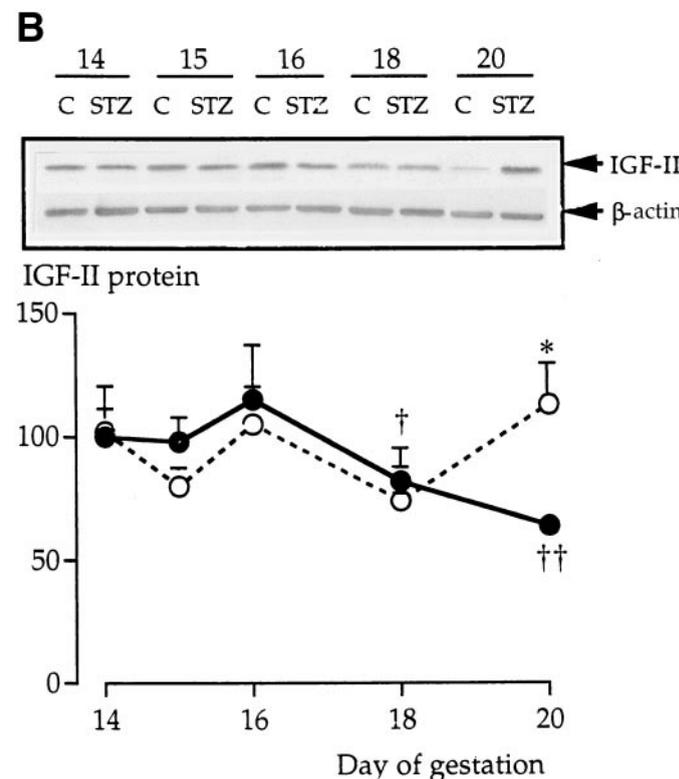
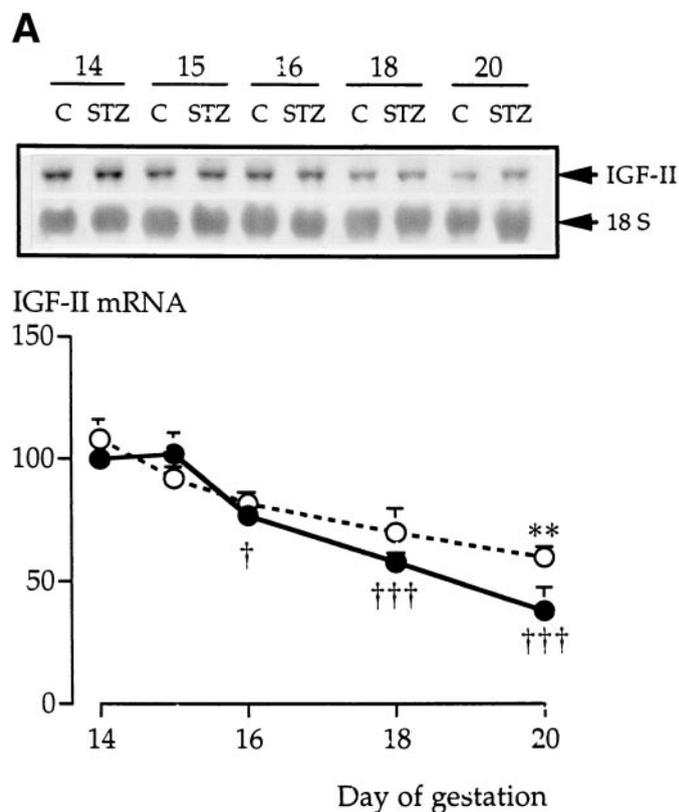


FIG. 2. Patterns of IGF-II mRNA (A) and protein (B) in the kidneys of fetuses (14–20 days of gestation) from normal (plain line) and diabetic (broken line) rats. Values are means \pm SE of five to six experiments. Densitometric results are expressed as percentage of the control (day 14) value after normalization for RNA loading on the basis of hybridization of 18 S rRNA and for protein loading by labeling with anti- β -actin antibody. †, ††, and †††: $P < 0.05$, 0.01, and 0.001 as compared with control (day 14) value. * and **: $P < 0.05$ and 0.01 as compared with age-matched control fetuses.

Although IGF-II/M6PR has been reported to play a role in transmembrane signal transduction (29–31), its function remains unresolved and controversial. The main function of this receptor in mammals is the transport of mannose-6-phosphate-containing lysosomal enzyme to lysosomes and the clearance of IGF-II from serum and tissue fluids by receptor-mediated endocytosis (25). Gene-targeting experiments also indicate that IGF-II/M6PR functions as a scavenger receptor by internalizing and degrading IGF-II (32–34). A truncated form of the IGF-II/M6PR cleaved from the cell-surface receptor may also bind a significant amount of IGF-II, thus functioning as an IGF-II specific binding protein regulating its bioavailability (35). This form seems to be most abundant in fetal circulation (35). It is also higher in the plasma of fetuses from diabetic women (35). We show that, at early stages of nephrogenesis, IGF-II/M6PR is localized in the nephrogenic zone, especially in undifferentiated mesenchymal cells. IGF-II mRNA is abundant in developing kidney in mice and humans and is mainly expressed in the mesenchymal cells (36,37). The colocalization of the receptor with the growth factor and the increase in the IGF-II/M6PR protein we observed in the kidneys of fetuses from diabetic mothers may thus reduce the bioavailability of IGF-II in this organ.

Several studies have reported that IGF-II is critical during fetal nephrogenesis. The development of the metanephros *in vitro* is prevented by adding anti-IGF-I and anti-IGF-II antibodies to the culture medium (19). However, using metanephric organ cultures and specific glomerular labeling, we showed that IGF-II stimulates the growth of the metanephros and the number of nephrons formed, whereas IGF-I only stimulates growth of the metanephros (38). It has also recently been shown that exogenous IGF-I does not increase the number of nephrons that has been reduced before the end of nephrogenesis by unilateral ureteral obstruction in neonatal rats (39). The present study shows that IGF-II mRNA and IGF-II protein are developmentally regulated and that their expressions decrease at the end of gestation, as the fetal kidney matures. Together, these data suggest that IGF-II is an autocrine/paracrine growth factor for mesenchymal cell differentiation.

The expression of IGF-II in the kidneys of fetuses from diabetic subjects increased at the end of gestation. This increase may be due, in part, to an increase in plasma IGF-II, which has been reported to occur in fetuses from diabetic rats and humans at the end of gestation (17,35,40). Such an increase in IGF-II would not be sufficient to restore the reduced number of nephrons caused by exposure to diabetes *in utero* (9), because it occurs concomitantly with the greatest increase in the IGF-II/M6PR and only at the end of nephrogenesis.

An increase in IGF-II/M6PR expression related to increased mRNA expression has also been shown in the kidney of diabetic adult rats (41–43). This might be of importance because the kidney is one of the most affected organs in respect to diabetic complications, and changes in the IGF-II/M6PR expression might be relevant to the development of such complications.

The mechanism whereby diabetes increases the expression of IGFs and IGF receptors is unknown. However, glucose *per se* has been shown to regulate the expression

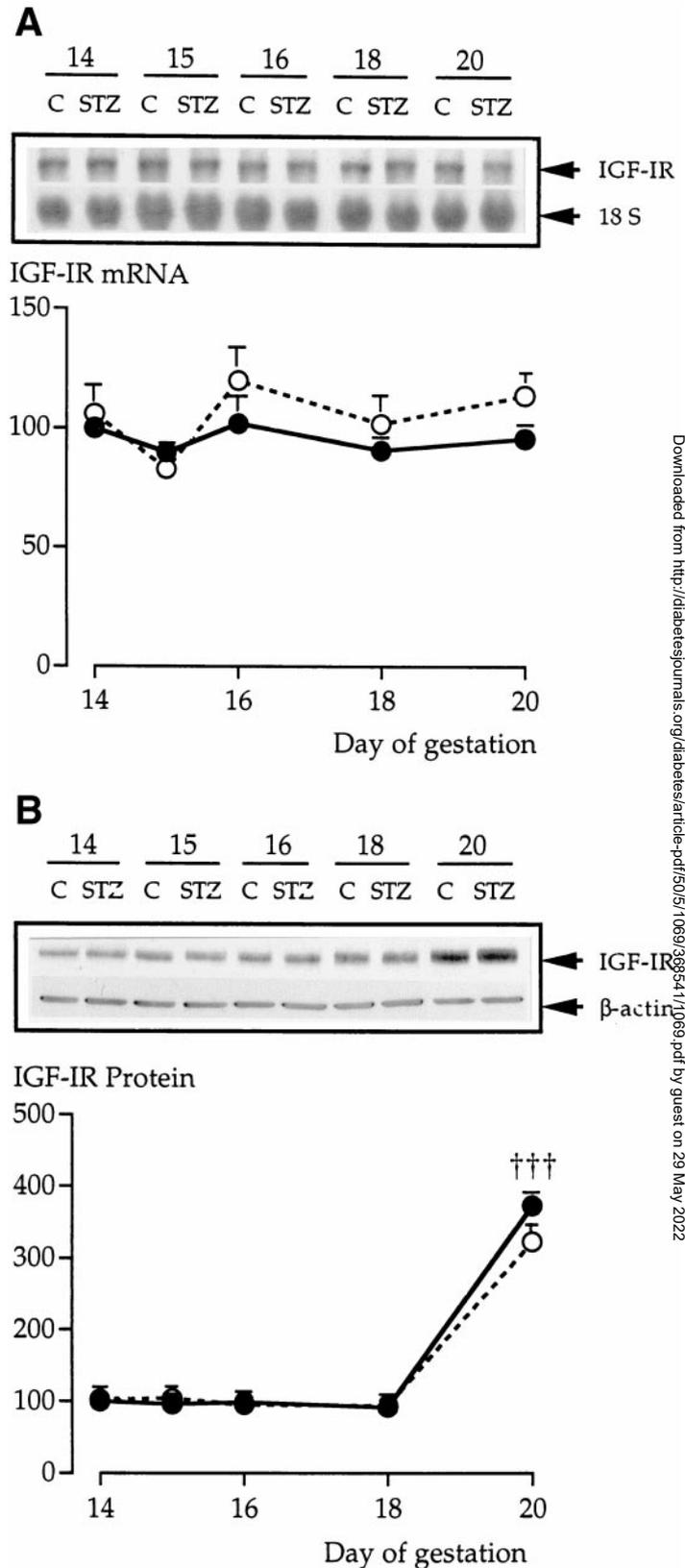


FIG. 3. Patterns of IGF-IR mRNA (A) and protein (B) in the kidneys of fetuses (14–20 days of gestation) from normal (plain line) and diabetic (broken line) rats. Values are means \pm SE of five to six experiments. Densitometric results are expressed as percentage of the control (day 14) value after normalization for RNA loading on the basis of hybridization of 18 S rRNA and for protein loading by labeling with anti- β -actin antibody. $\dagger\dagger\dagger P < 0.001$ as compared with control (day 14) value.

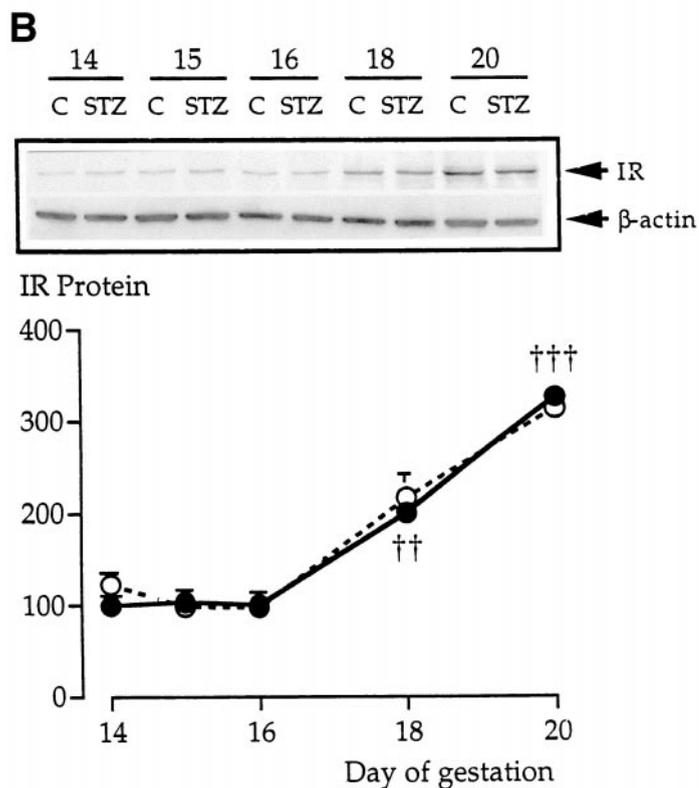
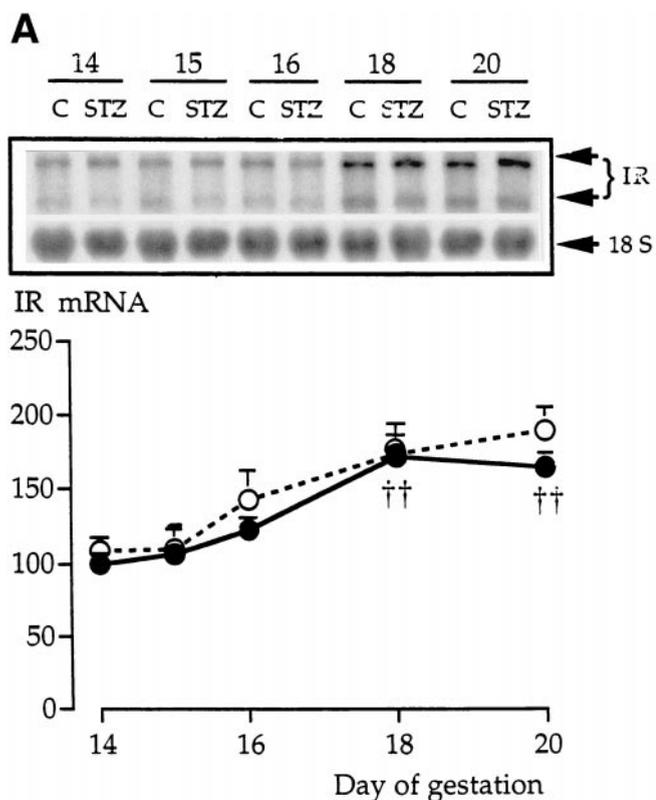


FIG. 4. Patterns of IR mRNA (A) and protein (B) in the kidney of fetuses (14–20 days of gestation) from normal (plain line) and diabetic (broken line) rats. Values are means \pm SE of five to six experiments. Densitometric results are expressed as percentage of the control (day 14) value after normalization for RNA loading on the basis of hybridization of 18 S rRNA and for protein loading by labeling with anti- β -actin antibody; mRNA values are the addition of the bands corresponding to the two transcripts. \ddagger and $\ddagger\ddagger$: $P < 0.01$ and 0.001 as compared with control (day 14) value.

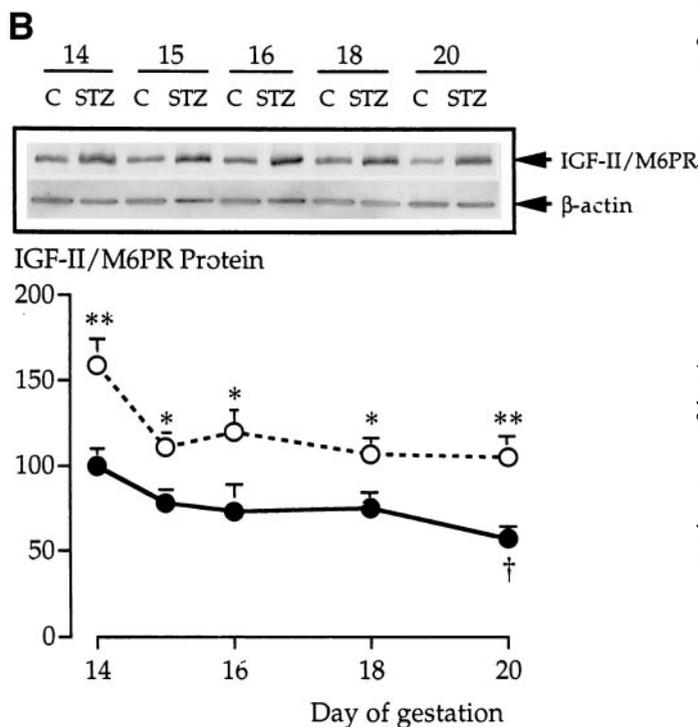
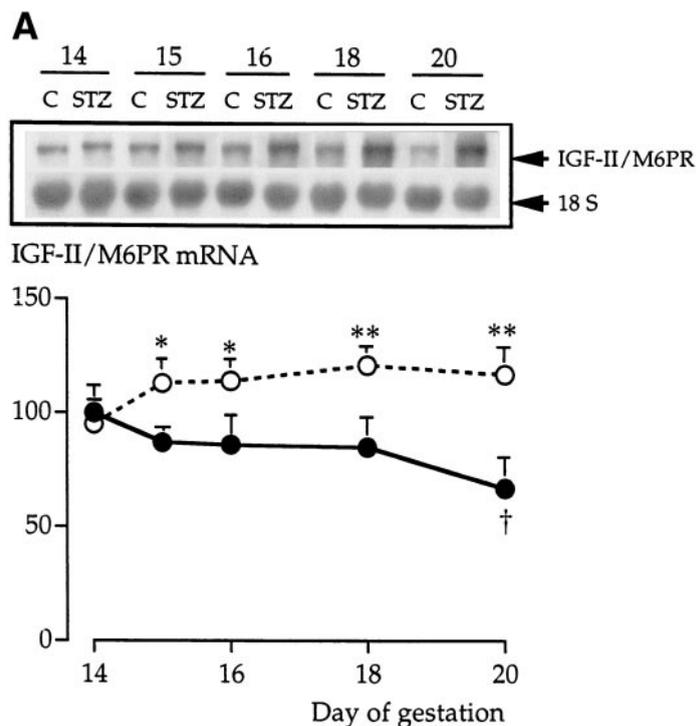


FIG. 5. Patterns of IGF-II/M6PR mRNA (A) and protein (B) in the kidneys of fetuses (14–20 days of gestation) from normal (plain line) and diabetic (broken line) rats. Values are means \pm SE of five to six experiments. Densitometric results are expressed as percentage of the control (day 14) value after normalization for RNA loading on the basis of hybridization of 18 S rRNA and for protein loading by labeling with anti- β -actin antibody. $\dagger P < 0.05$ as compared with control (day 14) value. * and **: $P < 0.05$ and 0.01 as compared with age-matched control fetuses.

of the IGF system in some in vitro studies. Goya et al. (44) have shown that glucose affects the transcription of the IGF-II gene in fetal rat hepatocytes. Similarly, increased IGF-II mRNA and IGF-IR mRNA, as well as an increased

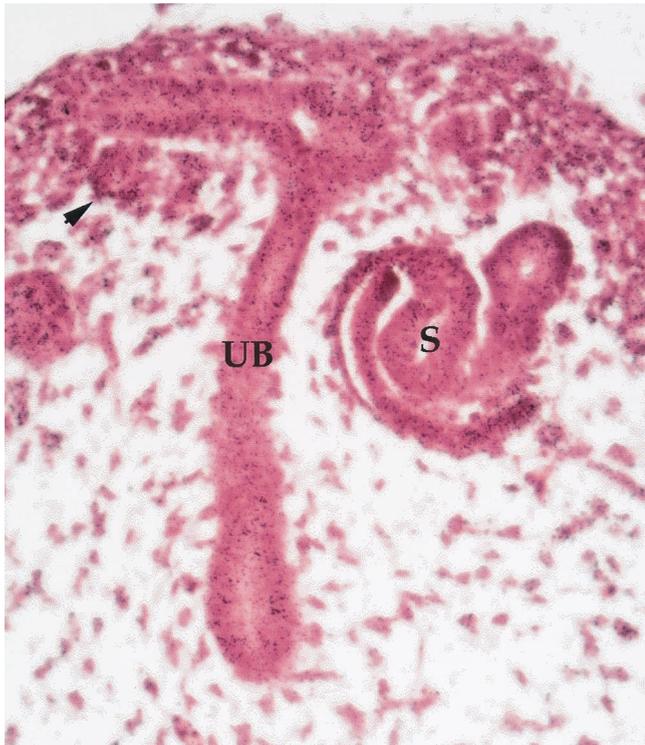


FIG. 6. Silver grains labeling of IGF-II/M6PR mRNA in the kidneys of fetuses on day 16 of gestation. In situ hybridization with ^{35}S -labeled IGF-II/M6PR probe followed by autoradiography hematoxylin-eosin counterstaining. UB, ureteric bud; S, S-shaped body.

number of IGF-II/M6PR, have been found in mesangial cells cultured in high-glucose medium (45). The same results were obtained with the insulin-secreting cell line RINm5F (46). These data support the idea that IGFs are encoded by genes belonging to the family of genes that are regulated by glucose. Daniel and Kim (47) have reported that transcriptional activator Sp1 binding is required for activation of the acetyl-CoA carboxylase promoter by glucose. But, the CACGTG-type E boxes are critical for the glucose response of the liver-type pyruvate kinase (48). Either the Sp1 binding sites or the CACGTG-type E boxes were found within the proximal promoter of the IGF-II/M6PR. This suggests that glucose regulates the transcription of this receptor.

We have shown for the first time that maternal diabetes causes an increase in the concentration of IGF-II/M6PR in the fetal kidney. Because the receptor is crucial for regulating the levels of free IGF-II ligand, this increase may lead to a decrease in IGF-II action. Because IGF-II plays a critical role in renal development, this may explain the altered nephrogenesis in fetuses of diabetic subjects.

ACKNOWLEDGMENTS

We thank Drs. MacDonald, Haugel, Clément, and Maitre and Professor Rotwein for kindly providing antibody and specific probes; J. Vilar and C. Lalou for critically reading the manuscript; and M.F. Belair for technical assistance with in situ hybridization.

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