Tissue-Specific Regulation of Insulin Receptor mRNA Levels in Rats With STZ-Induced Diabetes Mellitus

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In rats with STZ-induced diabetes mellitus, a reduction in insulin secretion is associated with increased insulin binding in the liver, muscle, fat, and kidney, but not in the brain. To test the hypothesis that tissue-specific modulation of insulin receptors (IRs) in STZ-induced diabetes occurs at the level of mRNA, IR mRNA levels were measured in the liver, kidney, and brain of Sprague-Dawley rats 15 days after intravenous administration of STZ (60 mg/kg body weight) and compared with those of control rats. Diabetic rats were either left untreated or given differing insulin regimens that were designed to achieve varying degrees of metabolic control. IR mRNA levels were measured by slot blot hybridization with a ^{32}P-labeled rIR probe and standardized by 28S ribosomal RNA determination. Hepatic IR mRNA levels were increased significantly in both untreated diabetic rats and in those that received low-dose (2 U/day) insulin therapy. In contrast, hepatic IR mRNA levels did not differ significantly from controls in those that received moderate doses of insulin (3–8 U/day) and were significantly less than controls in those that received the highest doses (6–10 U/day). Renal IR mRNA levels also were increased significantly in the untreated diabetic rats but not in those that received low- or moderate-dose insulin therapy, and were significantly less than controls in those that received the highest doses. A highly significant negative correlation was observed between the level of hepatic ($r = -0.84$, $P < 0.001$) and renal ($r = -0.64$, $P < 0.001$) IR mRNA, and the plasma concentration of insulin obtained at the time of death. No significant difference was observed in brain IR mRNA levels between untreated diabetic and control rats. Thus, in rats with insulin deficiency, modulation of insulin binding in the liver and kidney can be attributed, at least in part, to a change in steady-state IR mRNA levels. ***Diabetes*** **41:1113–18, 1992**

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STZ, streptozocin; rIR, rat insulin receptor; IR, insulin receptor; bp, base pair; SSC, sodium chloride-sodium citrate; SDS, sodium dodecyl sulfate; ANOVA, *analysis of variance*.

In rats with STZ-induced diabetes mellitus, a reduction in insulin secretion is associated with increased insulin binding in the liver (1–3), muscle (4,5), fat (6,7), and kidney (8,9), but not in the brain (10,11). This increase in insulin binding in tissues other than the brain has been attributed to up-regulation of IRs in response to the hypoinsulinemia that characterizes this model of diabetes. As previously reported by Okabayashi et al. (12), insulin can regulate the number of its receptors in cultured cells both by modulating their biosynthesis at the level of gene transcription and by influencing the rate of degradation/inactivation of the ligand-receptor complex. The purpose of this study was to test the hypothesis that the tissue-specific modulation of IRs in STZ-induced diabetes occurs as a consequence of changes in mRNA levels.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA), ranging in weight from 150–200 g, were maintained on standard rat chow and tap water ad libitum with 12-h light/dark cycles in a quiet environment. Diabetes mellitus was induced by intravenous administration of STZ (Sigma, St. Louis, MO), 60 mg/kg body weight, dissolved in sodium citrate buffer (0.1 M, pH 4.5) at a 20 mg/ml concentration immediately before use. Control rats, which were matched for age and weight at the time of STZ administration, received an equal volume of the medium. Rats were considered diabetic if blood glucose levels were ≥350 mg/dl 72 h after the injection.

Experiment 1. Diabetic rats were further divided into two subgroups: untreated rats ($n = 7$), and rats to which

human recombinant insulin (Humulin N, Lilly, Indianapolis, IN) was administered twice daily (0800 and 1600) in moderate doses (3–8 U/day) that were adjusted to prevent severe hyperglycemia ($n = 8$). In control rats ($n = 5$) and untreated diabetic rats, blood glucose levels were measured every other day by tail vein sampling (Accu-chek bG, Bio-Dynamics, Boehringer-Mannheim, Indianapolis, IN), whereas in the treated animals, insulin dosage was adjusted on the basis of daily determinations. Rats were killed by decapitation 14 days after administration of STZ, and trunk blood was collected to determine plasma glucose and insulin levels. The liver, kidney, and brain were removed quickly and frozen in liquid nitrogen for subsequent RNA isolation.

Experiment 2. This experiment was performed with the same protocol except that one of the diabetic subgroups received a fixed low dose of insulin (2 U/day, $n = 8$), and the other subgroup ($n = 8$), a higher dose (6–10 U/day), which was adjusted to achieve euglycemia. In this experiment, only liver and kidney tissues were obtained for IR mRNA measurements.

RNA isolation. Total RNA was isolated from frozen tissue by a modification of the guanidine thiocyanate method of Chirgwin, as described previously (13). Briefly, after storage at −80°C, the tissue was homogenized in a solution containing 4 M guanidine thiocyanate (Fluka Chemical, New York), 5 mM sodium citrate (pH 7.0), 0.5% N-lauroylsarcosine (Sigma), and 0.1 M β-mercaptoethanol (Sigma) in a ratio of 16 ml/g of tissue mass. Homogenization was performed using a polytron (Brinkmann Instruments, Westbury, NY). Total nucleic acid was precipitated by adding 2.5% vol of 1 M acetic acid, 50% vol of 100% ethanol, and incubation at −20°C for a minimum of 4 h, followed by centrifugation at 12,000 *g* at −10°C for 20 min. The pellet was drained briefly and resuspended in a solution containing 7.5 M guanidine hydrochloride (Fluka), 25 mM sodium citrate, and 4.5 mM dithiothreitol, in a ratio of 8 ml/g of tissue. Total RNA was precipitated as indicated above. The resultant RNA pellet was dissolved in sterile water, and quantified by UV absorbance at 260/280 nm. RNA integrity was verified by agarose gel electrophoresis.

Probe synthesis. A ^{32}P-labeled rIR probe was synthesized by nick-translation of the 1791 bp Pst 1 fragment of rIR-p16 encoding the 5' end of the rIR (from Barry J. Goldstein, Joslin Diabetes Center, Boston, MA) (14). Nick-translation was performed with the Du Pont-NEN Nick-Translation System (NEK-004Z) and ^{32}P-dCTP (3000 Ci/mmol, Du Pont-NEN, Boston, MA). Probe purification was performed by spin-column centrifugation using a G-25 Sephadex DNA purification Quick Spin column (Boehringer Mannheim, Indianapolis, IN).

mRNA analysis. To determine both the specificity of the rIR probe and the ideal hybridization conditions for its use, poly(A)$^+$ RNA was prepared using Hybond mAP messenger affinity paper (Amersham, Arlington Heights, IL) and subjected to agarose gel electrophoresis followed by transfer to nylon filters by Northern blotting. The filters were probed with ^{32}P-labeled rIR cDNA. In autoradiographs, two major bands characteristic of rIR mRNA were seen at 7.2 and 9.4 kilobases (Fig. 1).

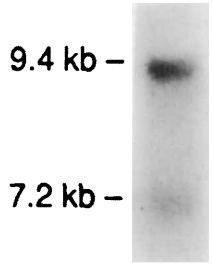

FIG. 1. Northern blot analysis of mRNA extracted from normal rat liver. The RNA was electrophoresed through a 1% agarose gel, transferred to a nylon filter, and hybridized with a ^{32}P-labeled rIR probe at 43°C. The probe was synthesized by nick-translation of the 1791 bp Pst 1 fragment of rIR-p16 encoding the 5' end of the rIR (>1644). The size of the two major bands detected is indicated in kilobases.

IR mRNA levels were quantified by slot blot hybridization. Briefly, serial dilutions of 4, 2, and 1 μg were prepared for each total RNA sample, and subsequently immobilized on 0.1 μm nitrocellulose using the Minifold II slot blot apparatus (Schleicher & Schuell, Keene, NH). Following immobilization, RNA was fixed to the membrane by baking at 80°C for 2 h. Membranes were prehybridized in a solution containing 50% formamide, 3X SSC, 10X Denhardt's, 10 mM Tris (pH 7.6), 400 μg/ml sheared denatured salmon sperm DNA, and 0.2% SDS for 2–4 h at 43°C. Hybridization was performed using fresh prehybridization solution, to which ^{32}P-labeled rIR probe was added at a final concentration of $2.5–3.0 \times 10^6$ cpm/ml of hybridization solution, and was incubated for 18–20 h at 43°C. After hybridization, the membranes were washed for 30 min at 43°C in 2X SSC/0.1% SDS, 0.5X SSC/0.1% SDS, 0.1X SSC/0.5% SDS, and finally in 0.1X SSC/ 0.5% SDS at 48°C. Autoradiographs were obtained by exposure to Cronex X-ray film (Du Pont-NEN) with an intensifying screen at −80°C for 9–11 days and were scanned with a laser densitometer (LKB 2202 Ultroscan, Piscataway, NJ). To assure equivalent loading conditions, duplicate blots were prepared and hybridized with a ^{32}P-labeled oligonucleotide complementary to bases 4011–4036 of human 28S ribosomal RNA. This represents the most abundant species of RNA in tissue and has been recommended as a means of assuring changes in expression of specific mRNAs (15). Probe purification was performed by spin-column centrifugation using a G-25 Sephadex DNA purification Quick Spin column. After prehybridization, hybridization was performed in a solution containing 4X SSC, 5X Denhardt's, 2% SDS, and 1% PPi, to which ^{32}P-labeled probe was added at a final concentration of 1.0×10^6 cpm/ml of hybridization solution, and was incubated for 18–20 h at 40°C.

Plasma insulin. Plasma insulin levels were determined by radioimmunoassay, using a double antibody technique (16). Guinea pig anti-rat insulin serum (Linco, Eureka, MO) was used as the first antibody. Rat insulin (Novo, Wilton, CT) was used as a standard. The tracer was ^{125}I-labeled receptor grade porcine insulin (Du Pont-NEN).

TABLE 1
Effect of STZ-induced diabetes mellitus with and without insulin treatment on body weight, plasma glucose, and insulin concentration in rats

Group	*n*	Body weight (g)	Plasma glucose (mg/dl)	Plasma insulin (ng/ml)
Experiment 1				
Control	5	272 ± 2	146 ± 3	7.2 ± 0.7
Diabetes	7	209 ± 7*	520 ± 40*	3.8 ± 0.4†
Diabetes + insulin (3–8 U/day)	8	262 ± 7	265 ± 44	8.6 ± 0.5
Experiment 2				
Control	8	308 ± 5	124 ± 3	5.2 ± 0.4
Diabetes + insulin (2 U/day)	8	236 ± 6*	440 ± 46*	1.5 ± 0.2*
Diabetes + insulin (6–10 U/day)	8	309 ± 6	94 ± 14	11.9 ± 1.1*

Values are means ± SE of the values obtained after intravenous STZ administration, 60 mg/kg, or medium. *n* = number of rats. Plasma glucose and insulin were measured on trunk blood at the time of death. Intergroup comparisons were done by ANOVA.
*$P < 0.001$ vs. control.
†$P < 0.005$ vs. control.

Statistical analysis. Data are presented as mean ± SE. Comparisons between groups were done by ANOVA, and the relationship between plasma insulin and IR mRNA was examined by linear regression analysis. Differences were considered statistically significant when $P < 5\%$.

RESULTS

Hyperglycemia occurred within 3 days of STZ administration and persisted thereafter in both the untreated diabetic rats (Table 1, experiment 1) and in those that received low-dose insulin therapy (Table 1, experiment 2; Fig. 2). Hyperglycemia was less severe in the subgroup that received moderate doses of insulin (Table 1, experiment 1) and was ameliorated completely in those that received the highest dose (Table 1, experiment 2; Fig. 2). Rats treated with the two highest doses of insulin gained weight rapidly, reaching values not significantly different from control animals at the end of the experiment period (Table 1, experiments 1 and 2; Fig. 2). Plasma insulin levels were reduced significantly in both the untreated and low-dose insulin treated animals, equivalent to controls in those that received moderate doses, and were significantly greater than controls in those that received the highest doses of insulin (Table 1).

Hepatic IR mRNA levels were increased significantly in both untreated diabetic rats (Fig. 3*A*) and in those that received low-dose insulin therapy (Fig. 3*B*). Both IR mRNA species were altered significantly by the treatment. In contrast, hepatic IR mRNA levels did not differ significantly from controls in those that received moderate doses of insulin (Fig. 3*A*) and were significantly less than controls in those that received the highest doses (Fig. 3*B*).

Renal IR mRNA levels also were increased significantly in the untreated diabetic rats (Fig. 4*A*) but not in those that received low (Fig. 4*B*) or moderate (Fig. 4*A*) doses of insulin. Renal IR mRNA levels were significantly less than control rats in those that received the highest dose of insulin (Fig. 4*B*).

A highly significant negative correlation was observed between the level of hepatic IR mRNA and the plasma concentration of insulin obtained at the time of death (Fig. 5*A*). A highly significant negative correlation also was observed between the level of renal IR mRNA and the plasma concentration of insulin (Fig. 5*B*).

No significant difference was observed in brain IR mRNA levels between untreated diabetic and control rats, but mRNA levels were significantly less in the subgroup that received moderate doses of insulin compared with controls (Fig. 6). Brain IR mRNA levels were not determined in the experiment in which high-dose insulin therapy was used.

In contrast to the change observed for IR mRNA levels in diabetic rats, levels of 28S ribosomal RNA did not differ significantly in the study groups. Thus the tissue-specific changes in IR mRNA observed in the diabetic rats is not likely to represent an artifact caused by changes in other RNA species.

DISCUSSION

The results of this study indicate that in rats with untreated STZ-induced diabetes, tissue-specific modulation of the IR occurs at the level of steady-state mRNA levels. In both the liver and kidney, the level of IR mRNA was increased significantly compared with nondiabetic controls, whereas no significant difference was noted in the brain. These findings accord with the results of studies of insulin binding in this model in which an increase in receptor number was demonstrated in the liver and kidney (1–3,8–9), but not in the brain (10,11) of diabetic rats. Homologous IR regulation by ambient insulin concentrations has been demonstrated in cultured cell lines such as human lymphocytes (17–19), and human (20) and chick hepatocytes (21). Studies in such in vitro preparations indicate that changes in the number of IRs can result from modifications of the rate of receptor synthesis, the rate of inactivation of the ligand-receptor complex, or a combination of these two factors. An accelerated rate of IR degradation with no modification of receptor biosynthesis was demonstrated in cultured hu-

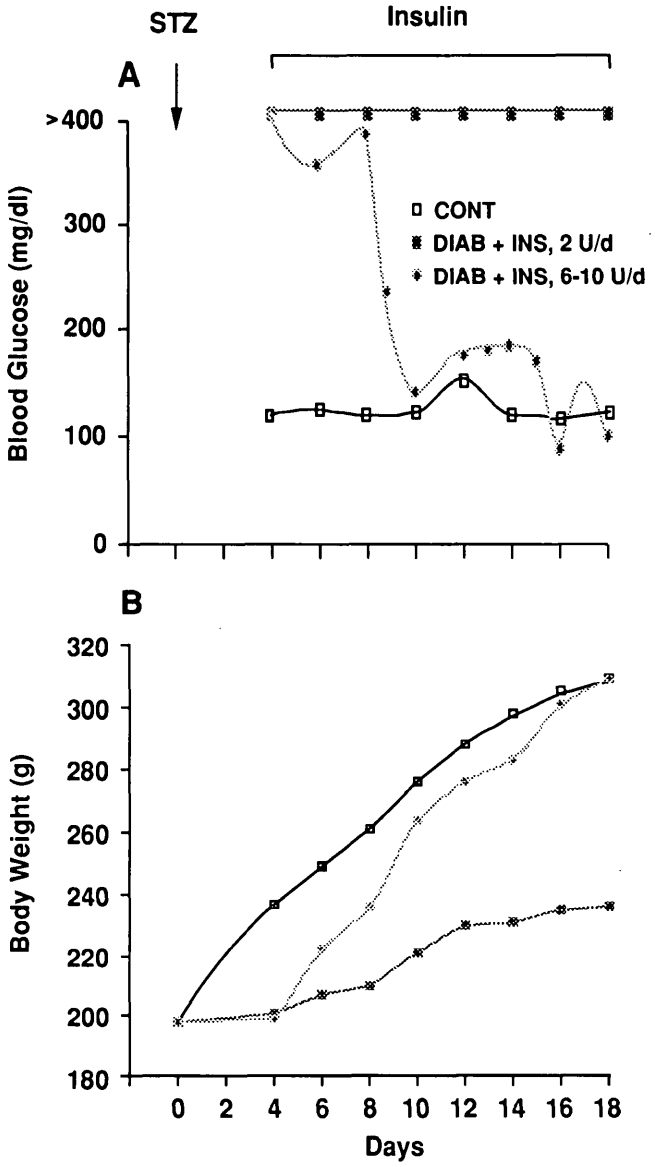

FIG. 2. Blood glucose concentration (*A*) and body weight (*B*) in experiment 2. Hyperglycemia occurred within 3 days of STZ administration and persisted thereafter in the subgroup of rats that were treated with a fixed low-dose (2 U/day) insulin regimen. Normalization of blood glucose was obtained in rats treated with a high-dose (6–10 U/day) regimen. In addition to maintaining normal glucose levels, the high-dose insulin treated subgroup gained weight normally.

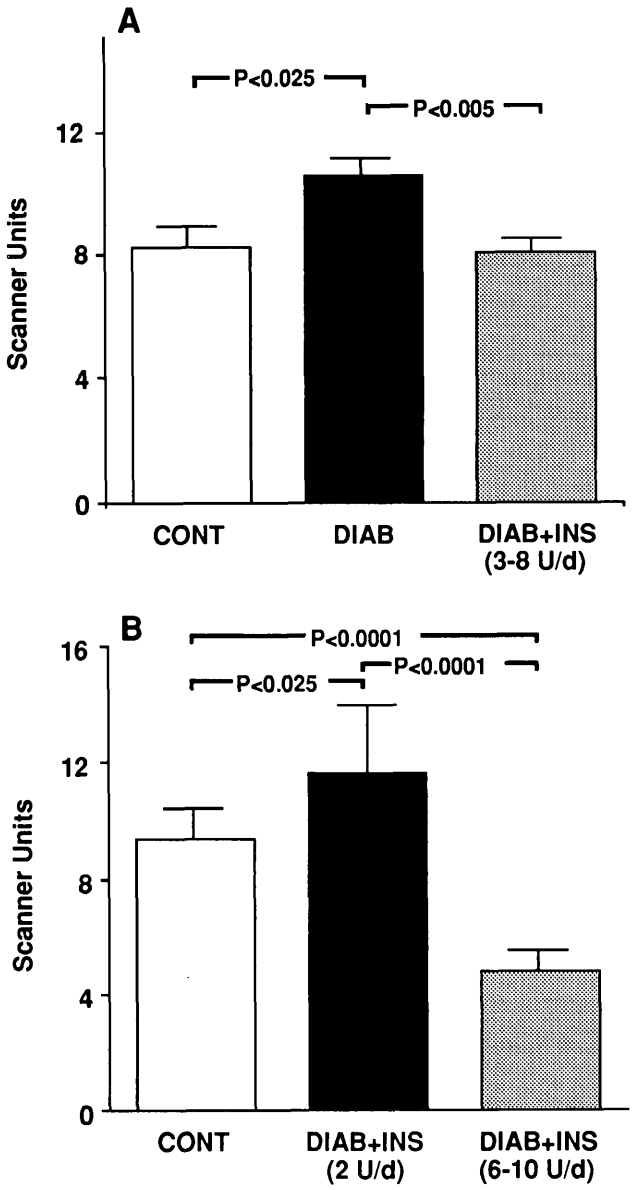

FIG. 3. Hepatic IR mRNA levels. IR mRNA levels were increased significantly in both untreated diabetic rats (experiment 1, *A*, [■]) and in those that received low-dose insulin therapy (experiment 2, *B*, [■]). In contrast, IR mRNA levels in diabetic rats did not differ significantly from control animals in the subgroup that received moderate doses of insulin (experiment 1, *A*, [▨]) and were significantly less than controls in those that received higher doses (experiment 2, *B*, [▨]).

man lymphocytes exposed to high levels of insulin (18). Similar results were observed in mouse fibroblasts (22).

In AR42J cultured pancreatic acinar cells, insulin-induced receptor down-regulation is caused by a combination of accelerated receptor degradation and decreased receptor biosynthesis; in these acinar cells, the increased synthetic rate appears to be caused, at least in part, by a decreased level of IR mRNA (12). Although this latter study indicates that excess insulin decreases IR mRNA in vitro, our experiments provide the first evidence that IR mRNA can be modulated in vivo. In rats with hypoinsulinemia induced by STZ injection, the inverse correlation between plasma insulin levels and IR mRNA in liver and kidney (Fig. 5) indicates that, even in vivo, insulin regulates the expression of the gene for its receptor.

Despite the increase in receptor number, rats with STZ-induced diabetes mellitus characteristically are insulin resistant. For example, insulin-induced glucose uptake in liver and muscle is reduced in such animals (4,6,23–26). This decrease in insulin action has been attributed to a decrease in IR autophosphorylation and tyrosine kinase activity (2,3,5,26–28), although abnormalities of these two enzymatic functions of the β-subunit of the receptor have not been invariable findings in

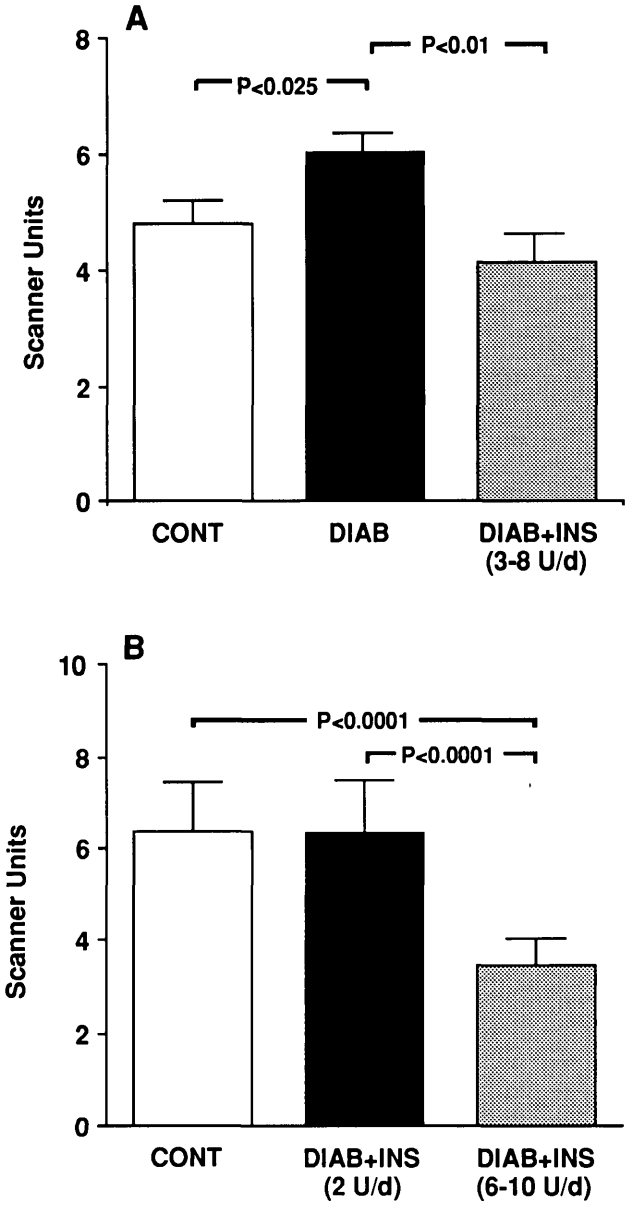

FIG. 4. Renal IR mRNA levels. IR mRNA levels were increased significantly in the untreated diabetic rats (experiment 1, *A*, [■]) but not in those that received low-dose insulin therapy (experiment 2, *B*, [■]). IR mRNA levels in diabetic rats did not differ significantly from controls in the subgroup that received moderate doses of insulin (experiment 1, *A*, [▨]) and were significantly less than controls in those that received higher doses (experiment 2, *A*, [▨]).

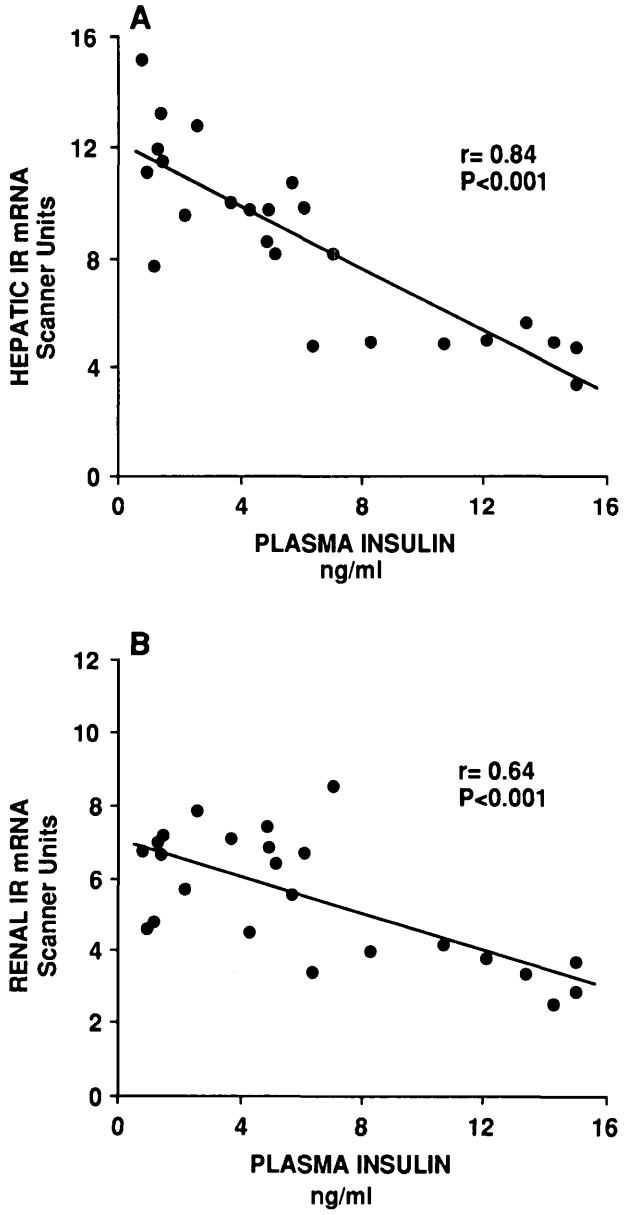

FIG. 5. Relationship between plasma insulin concentration and hepatic (*A*) and renal (*B*) IR mRNA levels in experiment 2. A highly significant negative correlation was noted between the level of both hepatic and renal IR mRNA and the plasma concentration of insulin. A similar negative correlation for both hepatic ($r = -0.54$, $P < 0.02$) and renal ($r = -0.48$, $P < 0.05$) IR mRNA and plasma insulin levels occurred in experiment 1 (data not shown).

STZ-induced diabetic rats (29–31). These apparent inconsistencies have been attributed to differences in the dose of STZ, the duration of diabetes, the tissues used, and the degree of purification of the membrane preparations (3). Nevertheless, the observation of decreased IR kinase activity in other insulin-resistant models (27,32,33) lends further support to an important role for this abnormality in the pathogenesis of insulin resistance.

In summary, in rats with insulin deficiency, the binding of insulin to its receptor in liver and kidney can be attributed, at least in part, to an alteration of IR steady-state mRNA levels. Thus, our results obtained in vivo indicate that regulation of IR gene expression can contribute to changes in insulin action together with modulation of receptor biosynthesis, degradation, and/or enzymatic activity.

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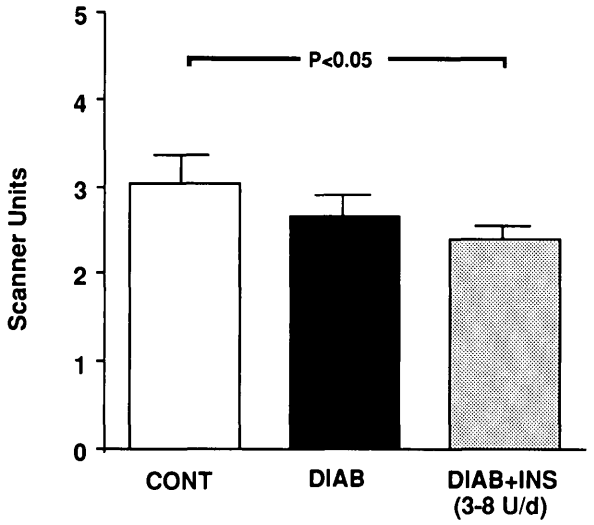

FIG. 6. Brain IR mRNA levels. No significant difference was observed between untreated diabetic (■) and control (□) rats, whereas IR mRNA levels were significantly less than controls in animals that received moderate doses of insulin (▦).

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