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Decreased Osteoblast Activity in Spontaneously Diabetic Rats

In Vivo Studies on the Pathogenesis

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Diabetes in both humans and rats is accompanied by low bone formation, which is presumably caused by serum-borne factors. To explore its pathogenesis, we carried out experiments in diabetic and nondiabetic BB rats, using plasma osteocalcin concentrations (OC) as a marker for osteoblast activity. In nondiabetic rats, the iv infusion of glucose (30%, 4 d) did not change OC; sc insulin infusion (4 U/d, 14 d) reduced OC by 27% ($p < 0.01$). In diabetic rats, OC were decreased from the first day of glycosuria (71 ± 5% of paired controls), declining exponentially to 24 ± 3% after 5 wk. Insulin infusion (1, 2, and 3 U/d, 14 d) produced gradual restoration of OC. OC were better correlated with insulin-like growth factor-I (IGF-I) than with insulin levels in these experiments. OC were dramatically increased 4 d after adrenalectomy (ADX) in all diabetic rats (73 ± 8 vs 22 ± 4 μg/L before ADX; $p < 0.001$), but not if corticosterone was administered. Ligand blotting of IGF binding proteins showed a marked decrease in two bands (44–49 and 32–35 kDa) 10–14 d after diabetes onset; the density of these bands was increased, but not normalized after ADX. Thus, decreased osteoblast activity is present from the onset of diabetes, is dependent on endogenous corticosterone, and cannot be reproduced by hyperglycemia in nondiabetic rats.

Key Words: Rat; diabetes; bone formation; osteocalcin; insulin; insulin-like growth factor-I (IGF-I); IGF binding proteins (IGFBPs); corticosterone.

Introduction

Diabetes mellitus is characterized by low bone formation. Bone histomorphometry in diabetic individuals as well as in spontaneously diabetic BB rats or streptozotocin (SZ)-induced diabetic rats indicates a marked decrease in the osteoblast and osteoid surface/perimeter and in the bone mineralization rate, on all (trabecular-endosteal, endocortical, and periosteal) envelopes of bone *(1–3)*. Electron microscopy of the endocortical bone surface shows that active, cuboidal osteoblasts are absent in SZ-induced diabetic rats, being replaced by flattened bone-lining cells exhibiting no evidence of matrix production *(4)*. Plasma concentrations of osteocalcin, a 5.7-kDa glycoprotein secreted by maturing osteoblasts *(5)*, are decreased in diabetic individuals of all age groups *(6)*; in severely diabetic BB rats, plasma osteocalcin is consistently <30% of the levels in age-matched controls *(2,7,8)*. Insulin treatment and pancreas transplantation in diabetic rats correct or even overcorrect the decrease in osteoblast/osteoid surface and plasma osteocalcin concentrations *(8,9)*.

The pathogenesis of low bone formation in diabetes is uncertain. Because serum osteocalcin levels are decreased at the time of diabetes diagnosis in children and adolescents, and revert to normal with intensive insulin treatment *(10)*, microangiopathy of bone is an unlikely explanation. Rather, one or more serum-borne factors are deemed to be responsible for this deficit, because sera from diabetic patients, particularly from those with poor metabolic control, impair collagen synthesis of human osteoblastic cells in vitro *(11)*. Diabetic rat sera also inhibit the collagen production by fibroblasts in vitro *(12)*; we found that serum from diabetic BB rats decreased the ^{3}H-thymidine incorporation in UMR-106 rat osteosarcoma cells (Verhaeghe, unpublished data). Which factor in diabetic serum would be responsible is unknown. Hyperglycemia was found to dampen the osteocalcin synthesis induced by $1,25(OH)_2D_3$ in human osteosarcoma MG-63 cells *(13)*. Second, hypoinsulinemia *per se* may be involved: human and rat osteosarcoma cell lines contain receptors for insulin and exhibit increased proliferation in the presence of insulin, and all osteoblastic cell lines examined to date show evidence of increased collagen or matrix synthesis after addition of physiological concentrations of insulin (reviewed in *14,15*). Third, the IGF-IGF binding protein (BP) system may be involved: sera from individuals with insulin-dependent diabetes (IDDM) *(11,16)* and SZ-induced diabetic rats *(17)* contain reduced concentrations of IGF-I, IGFBP-3, and

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IGFBP-4, but augmented concentrations of IGF-BP1 and IGFBP-2; IGF-I, possibly aided by IGFBP-3, stimulates osteoblastic proliferation and matrix synthesis, and inhibits matrix breakdown *(14,18)*, whereas IGFBP-1 inhibits IGF-I-induced osteoblastic proliferation *(19)*. Fourth, untreated or poorly treated diabetic individuals and severely SZ-induced diabetic rats appear to have increased circulating cortisol and corticosterone, respectively *(20,21)*; the excess of glucocorticoids may affect osteoblast function either directly or indirectly via changes in the IGFs or IGFBPs *(21–23)*. A role for the calciotropic hormones $1,25(OH)_2D_3$ and parathyroid hormone (PTH) as primary inducers of low bone formation in diabetes is more unlikely, however: although plasma concentrations of total $1,25(OH)_2D_3$ are decreased in male diabetic rats, we have shown that exogenous $1,25(OH)_2D_3$ in supraphysiological doses cannot restore normal osteoblast function *(24)*; PTH concentrations appear to be moderately decreased, but only after at least 9 d of SZ-induced diabetes *(3)*.

In this study, comprising a number of experiments, we explored some of the above possibilities. Plasma osteocalcin (OC) was used as an index of bone formation, since in our previous studies, this marker accurately reflected bone mineralization rate *(2,8)*. The role of insulin was evaluated by studying insulin and osteocalcin levels longitudinally after onset of diabetes, and by infusing insulin in diabetic and nondiabetic rats (Experiments 1 and 2). The role of hyperglycemia was assessed in an experiment in which hypertonic glucose was infused into nondiabetic BB rats (Experiment 3). The role of corticosterone was studied in adrenalectomized (ADX) diabetic rats with or without corticosterone replacement (Experiment 4). Concomitantly with osteocalcin measurements, we measured plasma concentrations of IGF-I and the vitamin D binding protein (DBP): the synthesis of these two proteins in the liver is strongly regulated by insulin *(25,26)*. In addition, the IGFBPs were analyzed by Western ligand blotting in some of the experiments because of their regulation by insulin and corticosterone *(17,21,23)*.

Results

Experiment 1: Longitudinal Effects of Diabetes in BB Rats

The body weight of the diabetic rats decreased linearly compared with nondiabetic littermates, and was significantly different from d 6 onward (Fig. 1). Plasma glucose levels were 2.4 times higher than controls on d 0 ($p < 0.001$), and remained between 2.6 and 3.1 times higher between d 2 and wk 5. Plasma insulin concentrations were 60 ± 30% of controls on d 0 ($p < 0.01$), but plummeted to 12 ± 4% on d 4 and remained <15% of controls thereafter. IGF-I concentrations were significantly lower than controls from d 6 (80 ± 6%) falling to 27 ± 6% ($p < 0.001$) after 5 wk. DBP levels were significantly different from controls on d 0 (88 ± 4%; $p < 0.05$), and then again from d 6 (82 ± 4%; $p < 0.01$) onward, dropping to 44 ± 5% ($p < 0.001$) after 5 wk. OC concentrations were significantly lower than in controls from d 0 (71 ± 5%; $p < 0.01$) and decreased exponentially to 24 ± 3% ($p < 0.001$) after 5 wk. OC concentrations in diabetic rats, expressed as a percentage of controls, were correlated with plasma insulin ($r = 0.39$; $p = 0.002$; $n = 62$) and IGF-I concentrations ($r = 0.72$; $p < 0.0001$; $n = 59$).

Plasma corticosterone levels were not different on d 0, d 14, or wk 5 (Table 1). The concentrations of corticosteroid binding globulin (CBG) were decreased after 5 wk (70 ± 10%), but not after 2 wk. This means that diabetes in the BB rat has an earlier and more profound effect on circulating DBP than on CBG concentrations (44 ± 5 vs 70 ± 10% of controls after 5 wk of diabetes, respectively). The calculated free corticosterone index was not significantly different at any time-point.

Western ligand blotting of IGFBPs showed three major bands (Figs. 2 and 3): the largest corresponded to protein(s) with a mol wt of 44–49 kDa (band 1), the second band was at 32–35 kDa (band 2), and a fainter band at 26 kDa (band 3). Band 1 almost certainly represents IGFBP-3, band 2 would correspond to IGFBP-1/IGFBP-2, and band 3 would correspond to IGFBP-4 *(17,21,27,28)*. On d 0 and 2, there was no difference in the density of each band compared with the paired control sample, or in the relative percentage of each band to the total IGFBP density. On d 10 and 14, however, bands 1 and 2 were much fainter ($p < 0.01$ with control sample), whereas band 3 showed no significant difference; the relative percentage of band 3 to the total IGFBP density was therefore slightly higher ($p = 0.06$) in diabetic rats.

Experiment 2: Effects of Insulin, Infused sc for 14 d in Diabetic and Nondiabetic BB Rats (Fig. 4)

Insulin infusions increased body weight gain in diabetic (1, 2, and 3 U/d) and nondiabetic (4 U/d) BB rats; the weight loss of diabetic rats was reversed by 1 U/d of insulin, and weight gain was supraphysiological with 2 and 3 U/d. Plasma glucose levels decreased linearly with increasing insulin dose in diabetic rats, but 3 U/d were necessary to normalize plasma glucose levels fully; insulin (4 U/d) had

Fig. 1. *(opposite page)* Longitudinal effect of diabetes in BB rats on body weight, and plasma glucose, insulin, IGF-I, DBP, and OC concentrations. Number of pairs was between 7 and 9 for d 0–14, and 15 for wk 5. The rats studied after 5 wk are the same (saline-infused control and diabetic rats) as in Fig. 4. Results are presented as the percent of the value obtained in the paired nondiabetic littermate. If the results showed a significant ($p < 0.05$) correlation with diabetes duration, the regression curve that best fitted the data is presented. For body weight, the regression was linear ($y = 92 - 0.77\times$ [d]; $R^2 = 0.53$; $p < 0.0001$). For IGF-I and DBP, the best regression was a polynomial quadratic equation ($R^2 = 0.61$ and $R^2 = 0.47$, respectively; $p < 0.0001$). For osteocalcin, an exponential regression curve was obtained ($y = 67 \cdot e{-}0.0346\times$; $R^2 = 0.62$; $p < 0.0001$). Statistical differences between diabetic and control rats were assessed by paired *t*-tests, as described in the Results.

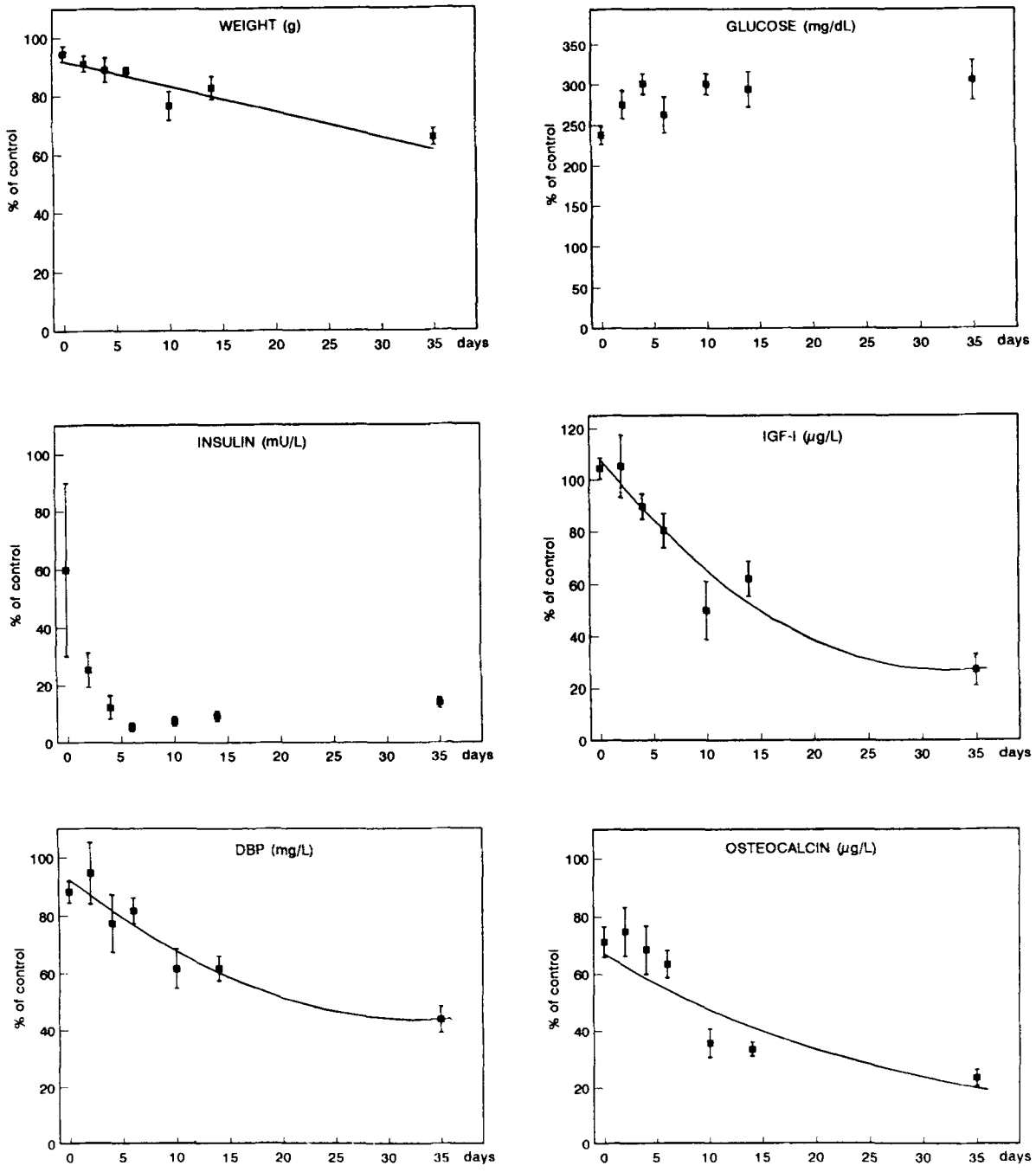

Fig. 1

Table 1
Plasma Concentrations of Corticosterone, CBG, and the "Free Corticosterone Index" At and After Onset of Diabetes in BB Rats[a]

	Total corticosterone, μg/dL	CBG, mg/L	Free corticosterone index
D 0 (n = 9)	105 ± 5	102 ± 5	104 ± 7
D 14 (n = 8)	111 ± 18	107 ± 5	103 ± 16
5 wk (n = 15)	89 ± 14	70 ± 10*	154 ± 29

[a]The data were expressed as the percent of the paired nondiabetic littermate. The free corticosterone index was calculated as the corticosterone/CBG molar ratio. The "5 wk" rats are the same as the saline-treated rats in Fig. 4. Statistical analysis was performed by paired t-tests between diabetic and nondiabetic littermates: *p = 0.008. Corticosterone was measured by RIA (*see* Materials and Methods); similar results were obtained using a competitive protein binding assay (data not shown).

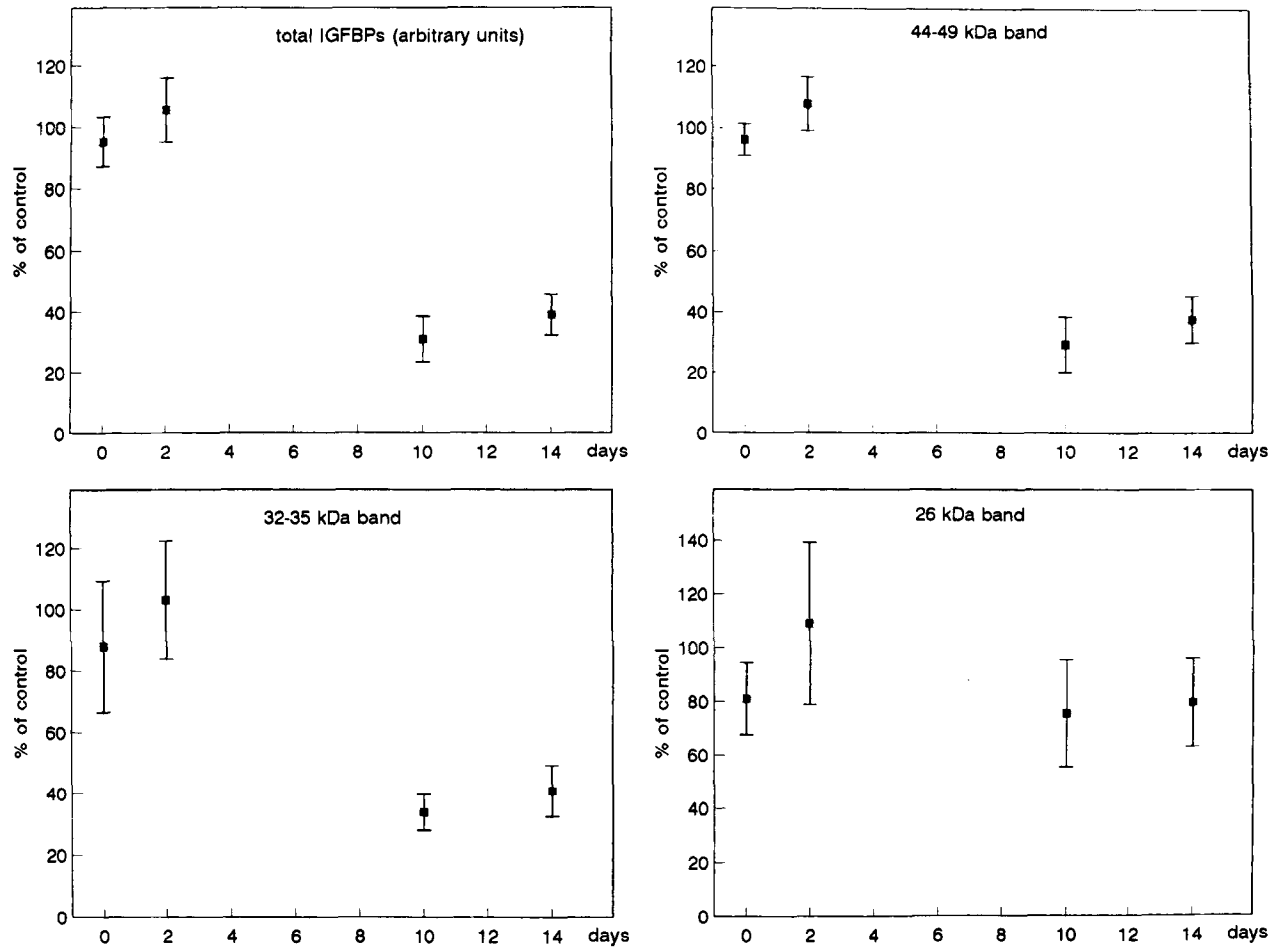

Fig. 2. Longitudinal effects of diabetes in BB rats on IGFBPs, as evaluated by Western ligand blot analysis. Number of pairs as in Fig. 1, only sera from d 0, d 2, d 10, and d 14 were examined. Results are presented as the percent of the value obtained in the paired nondiabetic littermate; the density of the bands was calculated as explained in Materials and Methods. Statistical differences were assessed by paired *t*-tests, as described in the Results.

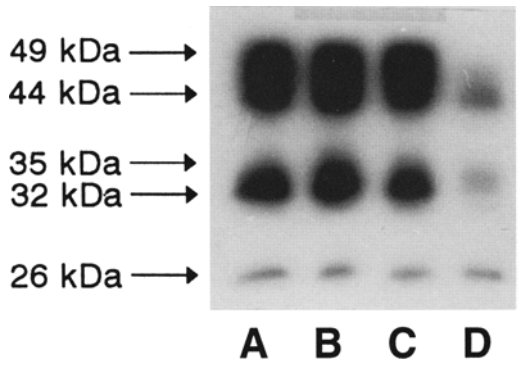

Fig. 3. Typical ligand blot of a control and diabetic rat plasma sample on d 0, and on d 14. **A** = control d 0, **B** = control d 14, **C** = diabetic d 0, **D** = diabetic d 14.

no effect on plasma glucose levels in nondiabetic rats. Plasma insulin concentrations increased with 4 U/d insulin in nondiabetic rats; plasma insulin levels were normalized by 2 U/d of insulin in diabetic rats, and were supraphysiological with 3 U/d. Plasma IGF-I concentrations did not alter with 4 U/d of insulin in nondiabetic rats; 2 U/d of insulin were sufficient to normalize IGF-I levels in diabetic rats, but 3 U/d of insulin did not produce supraphysiological levels. OC concentrations increased steadily with insulin treatment in diabetic rats, but 3 U/d were necessary for normalization; 4 U/d of insulin in nondiabetic rats decreased OC from 89 ± 5 to 65 ± 6 μg/L. There was no difference between the plasma corticosterone concentrations of saline-infused and insulin-infused nondiabetic rats (data not shown). The correlation coefficient between OC and insulin concentrations in this experiment was 0.57 ($p < 0.001$; $n = 65$) and between plasma osteocalcin and IGF-I 0.77 ($p < 0.001$; $n = 54$).

Experiment 3: Effects of iv Glucose Infusion in Nondiabetic BB Rats (Table 2)

Glucose (30%), infused intravenously for 96 h, increased plasma glucose and insulin levels, and there was a slight increase in DBP levels (IGF-I was not measured in this experiment); however, there was no effect on OC concentrations.

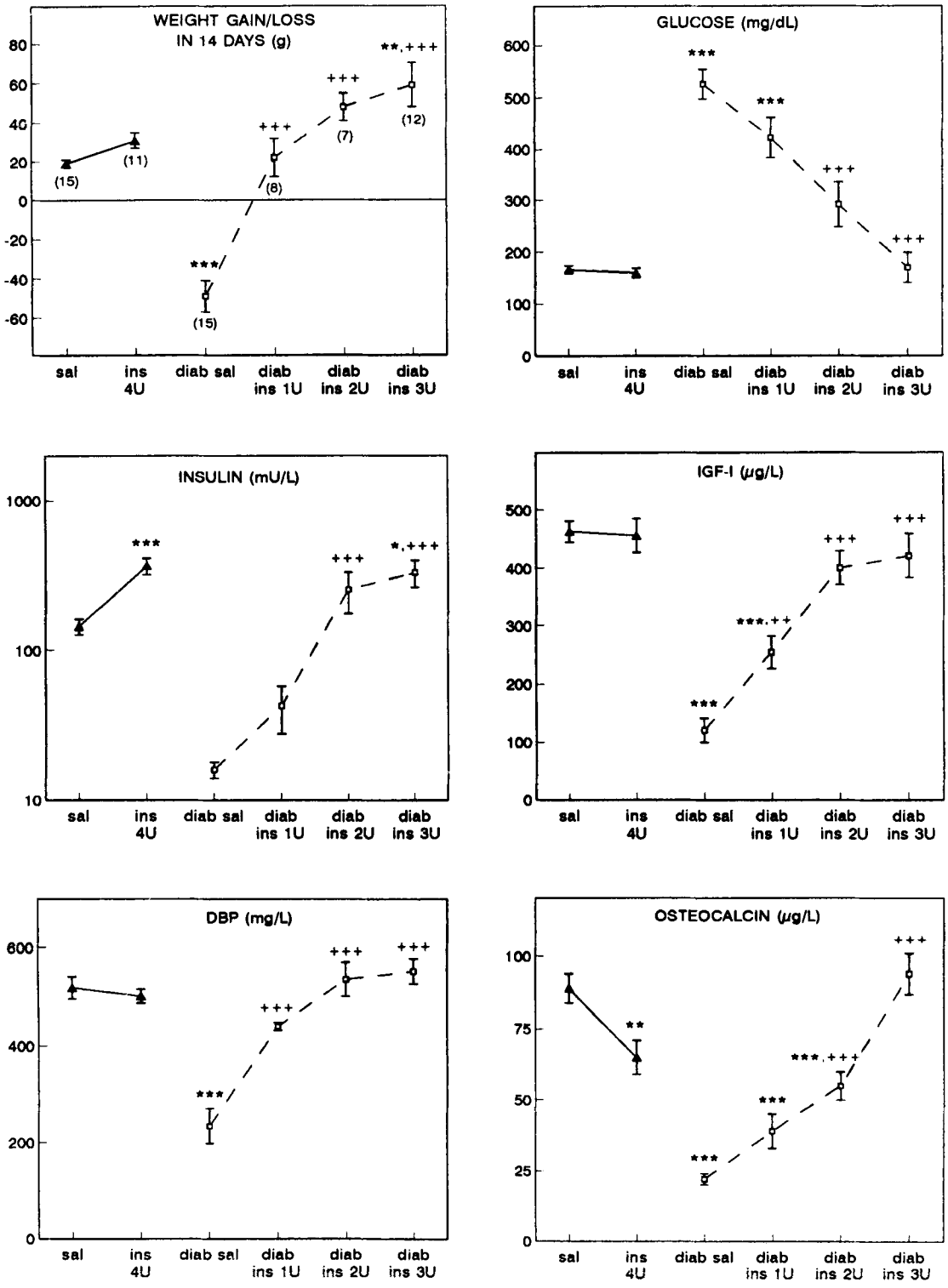

Fig. 4. Effect of insulin, infused sc via Alzet miniosmotic pumps during 14 d, on body weight change, and plasma glucose, insulin, IGF-I, DBP, and OC concentrations. Statistical analysis: one-way ANOVA was carried out to detect overall differences, and if $p < 0.05$ was followed by Tukey's multiple comparison test; differences shown by these tests between groups were then assessed by unpaired t-tests: * vs control saline (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); +: vs diabetic saline (+: $p < 0.05$; ++: $p < 0.01$; +++: $p < 0.001$). The insulin data are presented in a logarithmic scale; all other data are in a linear scale.

Experiment 4: Effects of ADX with or Without Corticosterone Treatment in Diabetic BB Rats

Plasma corticosterone was undetectable (<1 µg/dL in 6 rats) or very low (2.5 µg/dL in 1 rat) in the ADX rats, but returned to physiological (2 rats) or supraphysiological (5 rats) concentrations with corticosterone treatment (15 mg/day, sc) (Fig. 5). In all seven ADX diabetic rats, OC concentrations increased 4 d after ADX from 22 ± 4 µg/L to 73 ± 8 µg/L

Table 2
Effect of Hypertonic Glucose (30%)
Infused Intravenously During 96 h in 10 Nondiabetic BB Rats on OC[a]

	Before	After infusion	
Body weight (g)	—	421 ± 16	
Plasma glucose (mg/dL)	145 ± 10	344 ± 45	($p = 0.003$)
Insulin (mU/L)	45 ± 7	381 ± 114	($p = 0.02$)
DBP (mg/L)	462 ± 23	532 ± 24	($p = 0.08$)
OC (μg/L)	61 ± 6	69 ± 11	($p = 0.37$)

[a]A blood sample was taken before infusion and 96 h after infusion under anesthesia. Statistical analysis was performed by paired *t*-tests.

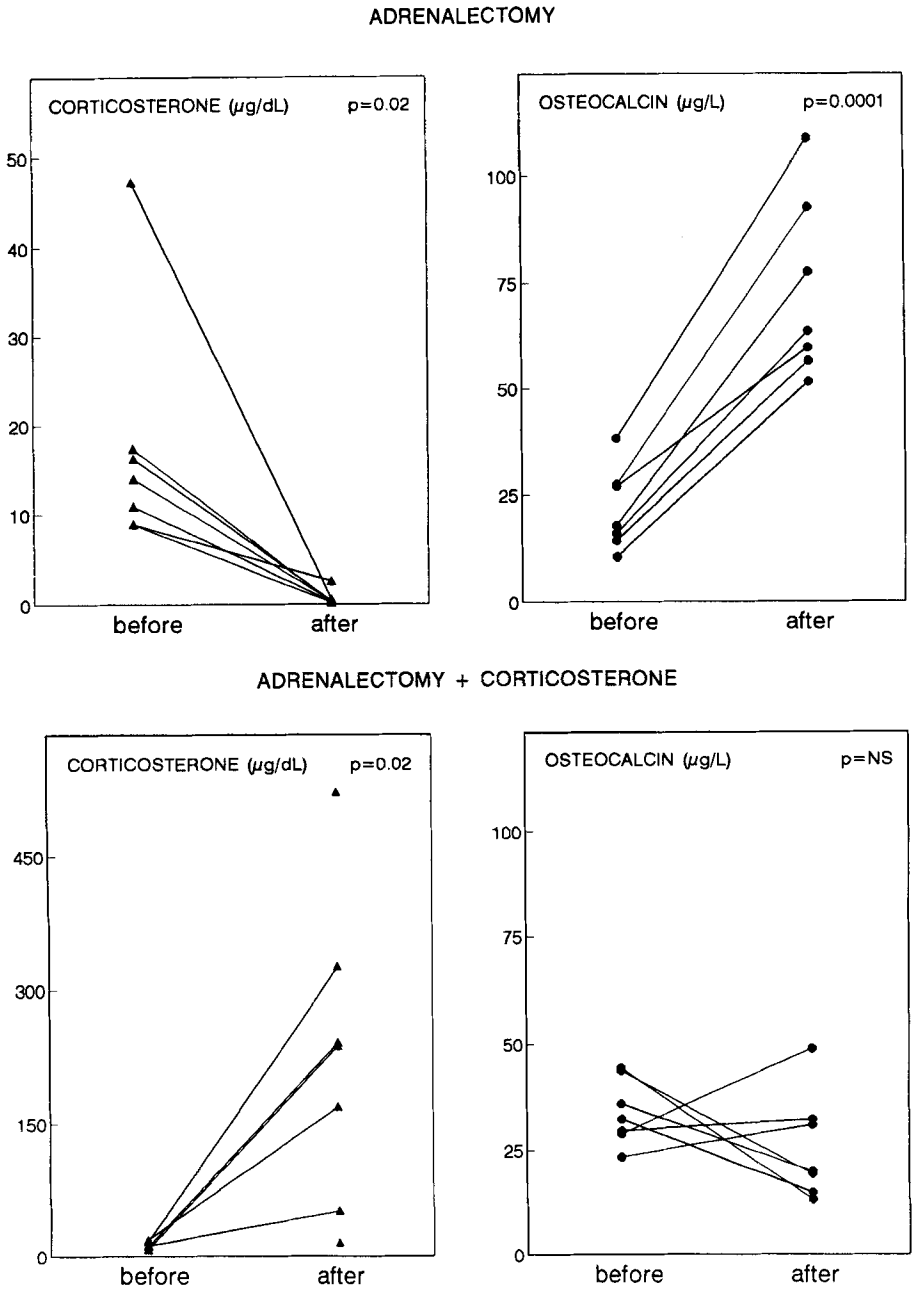

Fig. 5. Effects of ADX and ADX with corticosterone treatment (15 mg/d, sc) on plasma corticosterone and OC concentrations. The figure shows the individual data before ADX and 96 h after ADX. Paired *t*-tests were used for statistical analysis. NS, not significant.

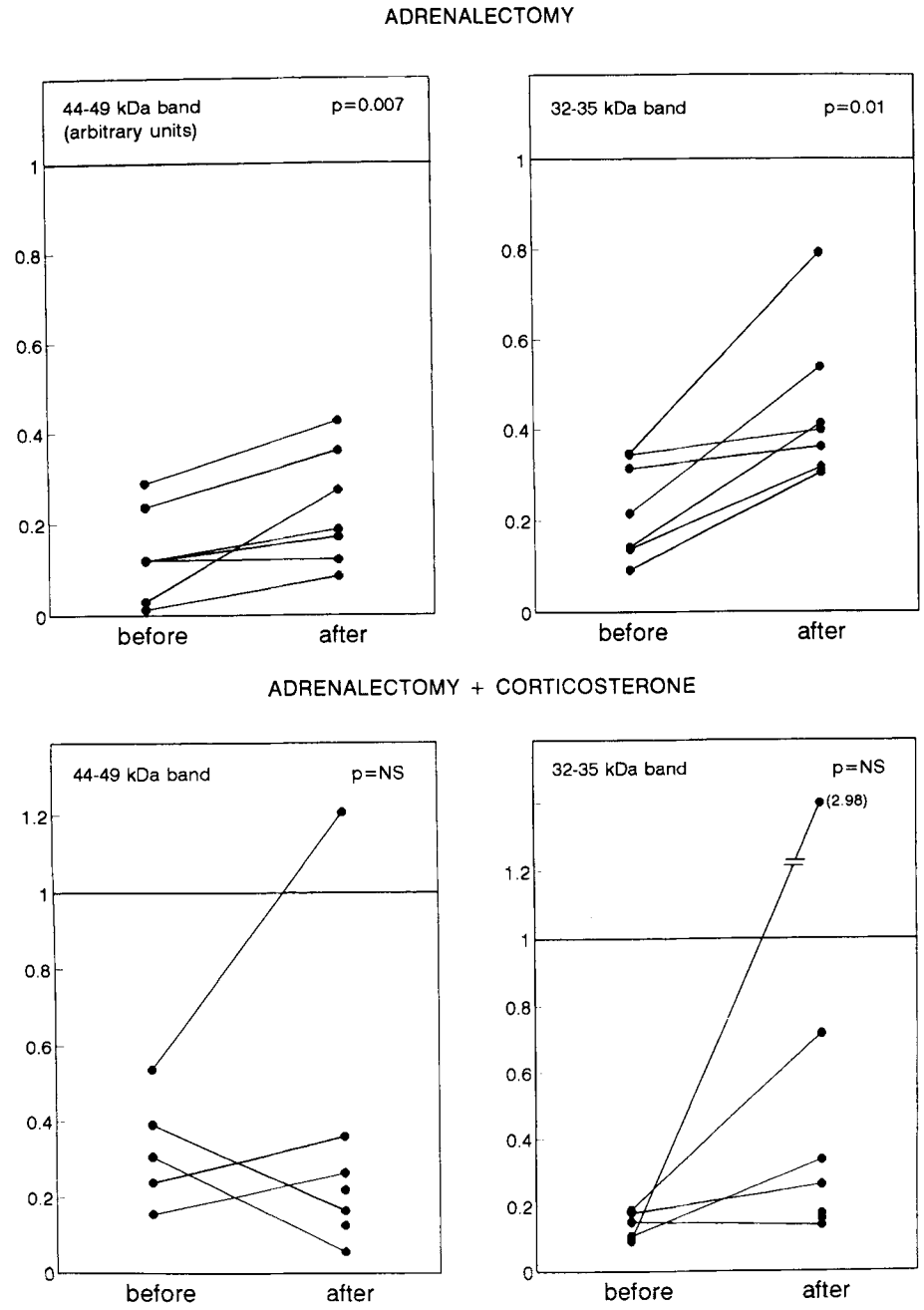

Fig. 6. Effect of ADX and ADX with corticosterone treatment on the density of band 1 (44–49 kDa) and band 2 (32–35 kDa). The figure shows the individual data before ADX and 96 h after ADX. Paired *t*-tests were used for statistical analysis. NS, not significant.

($p = 0.0001$), but not after ADX with corticosterone treatment (from 34 ± 3 µg/L to 25 ± 5 µg/L; p = NS).

Because it has been reported that ADX and corticosterone treatment in diabetic rats modulate the concentrations of the IGFBPs *(21,23)*, Western ligand blotting was carried out in serum samples before and after ADX. Since diabetic rats showed decreased density of bands 1 (44–49 kDa) and 2 (32–35 kDa), we specifically looked at these bands. Figure 6 shows that in most diabetic ADX rats, there was an increase in the density of these two bands, as well as in the 26-kDa band ($p = 0.005$; data not shown); however, they remained well below control values. In contrast, although two serum samples before ADX + corticosterone were unavailable for analysis, only one rat appeared to show an increase in band 1; the overall change was not significant.

Discussion

The experiments of this in vivo study indicate that impaired osteoblast function, assessed by OC concentrations, in spontaneously diabetic rats:

1. Is already present on the first day of glycosuria, during which plasma glucose levels have been shown to rise abruptly in longitudinal studies in BB rats (29);

2. Cannot be mimicked, however, by hyperglycemia for 4 d in nondiabetic rats; and
3. Is dependent on endogenous corticosterone.

OC concentrations were lower than in nondiabetic littermates from the onset of glycosuria and decreased exponentially with longer diabetes duration. The former finding confirms data in diabetic children or adolescents in whom low OC levels have been measured at first diagnosis, which reverted to normal with intensive insulin treatment *(10)*. Although in vitro results have shown that hyperglycemia inhibited the $1,25(OH)_2D_3$-induced OC secretion by osteoblastic cells *(13)*, Experiment 3 indicates that hyperglycemia in nondiabetic rats did not change OC levels. Clearly, diabetic serum is dissimilar to hyperglycemic-hyperinsulinemic control serum, but if hyperglycemia *per se* would be "toxic" for osteoblast activity, it should have resulted in lower OC levels after 96 h. Experiment 4 indicates that low bone formation in diabetic rats is dependent on endogenous corticosterone, because their low OC concentrations were normalized after ADX, but not after ADX with concomitant corticosterone treatment. Apart from insulin treatment *(8)* and pancreas transplantation *(9)*, which reverse the diabetic condition, ADX is the second experimental method—together with tetracycline treatment *(4)*—to succeed in stimulating osteoblast activity without restoring normoglycemia (plasma glucose before and after ADX: 620 ± 20 and 542 ± 28 mg/dL; $p = 0.09$). The mechanism of action of tetracycline has been attributed to its inhibition of collagenase activity, which was found to be higher in the gingiva of diabetic rats *(30)*. It is likely that osteoblastic collagenase activity is also increased in diabetic rats: indeed, the presence of inactive bone-lining cells on the endocortical surface *(4)* and the rapid changes in OC levels from the onset of diabetes suggest that osteoblasts are functionally suppressed. In diabetic humans, the relative decrease in OC levels is more pronounced than that of earlier biochemical markers of osteoblast differentiation *(6)*. ADX in normal male rats was found to stimulate morphometric and biochemical parameters of bone formation, in particular, the mineral apposition rate *(31)*, which reflects individual osteoblast function rather than osteoblast recruitment, and OC levels *(32)*, which also reflect osteoblast maturation *(5)*; however, corticosterone excess dose-dependently suppressed osteoblast function and recruitment *(31)*. The osteocalcin gene promoter contains both a vitamin D- and a glucocorticoid-responsive element *(5)*; interestingly, although OC levels were only minimally stimulated by high doses of exogenous $1,25(OH)_2D_3$ in diabetic BB rats *(24)*, OC remained responsive to glucocorticoids. Decreased osteoblast function in diabetic BB rats was not owing to circulating corticosterone excess, indicating that corticosterone plays a permissive rather than a direct role in low bone formation of diabetic rats. However, studies in SZ-induced diabetic rats have found increased serum total corticosterone *(20)*. The adrenocortical function of diabetic BB rats should therefore be further explored.

OC concentrations in Experiments 1 and 2 were correlated with both insulin and IGF-I concentrations; however, the correlation coefficient in both Experiments was higher for IGF-I than for insulin. Rat osteoblastic cells contain receptors for insulin and IGF-I *(14,15)*. We have previously reported that plasma IGF-I, which reflects the hepatic IGF-I production, is well correlated with OC in diabetic rats *(7)* as well as in ovariectomized rats treated with 17β-estradiol or/and recombinant human IGF-I *(33)*. In vitro, IGF-I inhibits the interstitial collagenase activity of osteoblasts obtained from fetal rat calvariae *(18)*. However, plasma IGF-I remained within normal limits 0–4 d after onset of diabetes, whereas OC was decreased from day 0. Insulin deficiency itself could be related to low bone formation in early diabetes, but other results were not compatible with a direct effect of insulin on osteoblast function. Whereas plasma insulin levels plummeted to <15% of controls by d 4, OC concentrations fell more gradually from $71 \pm 5\%$ of controls to $24 \pm 3\%$ after 5 wk. Exogenous insulin infusion or endogenous hyperinsulinemia provoked by hyperglycemia in nondiabetic rats did not stimulate OC; on the contrary, OC levels were decreased by 27% in insulin-infused rats. Low OC concentrations have also been reported in hyperinsulinemic Wistar fatty diabetic rats, as well as in hyperinsulinemic diabetic patients and hyperinsulinemic infants of diabetic mothers *(15)*. The addition of supraphysiologic doses of insulin to diabetic rat serum in vitro did not reverse its inhibitory effect on collagen synthesis by fibroblasts, whereas in vivo treatment proved to be effective *(12)*. To assess the effects of insulin on osteoblast function, euglycemic hyperinsulinemic clamp studies would be ideal, but are not feasible for a duration of more than 24 h.

Our Western ligand blot data indicated a decrease in both 44–49 kDa IGFBPs (band 1, corresponding to IGFBP-3) and 32–36 kDa IGFBPs (band 2, probably corresponding to IGFBP-1 and IGFBP-2), but not 26-kDa IGFBPs (band 3, probably corresponding to IGFBP-4) after 10–14 d of diabetes; ADX increased, but did not normalize the density of these bands. This confirms previous data by Luo and Murphy, who also reported a decrease in band 1 (39–42 kDa) and band 2 (28–30 kDa), but no change in band 3 (24 kDa), after 1 mo of diabetes in sera of BB rats; immunoblotting showed normal levels of IGFBP-1, whereas Northern blots of liver tissue showed decreased IGFBP-3, but increased IGFBP-1 and IGFBP-2 mRNA *(27)*. In SZ-induced diabetic rats, however, an increase in band 2 and a decrease in band 3, which were normalized by ADX, have been reported using ligand blots *(21)*; immunoblot data showed that IGFBP-1 is positively correlated and IGFBP-4 negatively correlated with corticosterone levels in SZ-induced diabetic rats *(23)*. These conflicting ligand blot results between spontaneously diabetic and SZ-induced

diabetic rats should be further examined using two-dimensional gel electrophoresis *(34)*, immunoblotting, specific radioimmunoassays of IGFBPs *(28)*, and IGFBP protease activity. Another promising avenue is to study the osteoblastic gene expression of the IGFBPs in diabetic rats: osteoblastic IGFBP-1 expression has recently been shown to be inhibited by insulin, but stimulated by glucocorticoids *(35)*. It is therefore possible that osteoblastic IGFBP-1 gene expression is switched on in diabetic, but not in ADX diabetic rats, and that this overexpression of IGFBP-1 would then suppress normal osteoblastic function, as has been shown in MG-63 osteosarcoma cells *(19)*.

Materials and Methods

Animals and Experiments

Male spontaneously diabetic BB rats (BB/pfd) were used for these studies. The Leuven BB rat colony is inbred since 1983 with a diabetes incidence of about 30%. Diabetes was diagnosed by the presence of glycosuria, which invariably corresponds to a plasma glucose level of >300 mg/dL. All rats were fed a laboratory rat chow containing 0.9% calcium, 0.62% phosphate, and 2000 IU vitamin D_3/kg (Hope Farms, Woerden, the Netherlands). All experiments described below were approved by the ethical committee of the Leuven Proefdierencentrum (pfd).

In Experiment 1, the rat colony was checked daily for the presence of glycosuria, and blood was sampled from groups of rats on the first day of glycosuria (d 0), and 2, 4, 6, 10, and 14 d thereafter; control rats were nondiabetic littermates. Blood was sampled from the abdominal aorta under pentobarbitone anesthesia (60 mg/kg, ip).

In Experiment 2, diabetic and nondiabetic BB rats received saline or insulin subcutaneously for 14 d using an Alzet miniosmotic pump (model 2002) with continuous insulin delivery, inserted under mild ether anesthesia. The diabetic rats had a diabetes duration of about 3 wk, and were given saline, and 1, 2, or 3 U of insulin (Actrapid HM, 500 IU/mL, Novo Nordisk A/S, Bagsvaerd, Denmark). We have previously shown that 3 U of insulin fully corrects the parameters of low bone formation in diabetic rats *(8)*. Control rats were infused with 4 U of insulin, because we found in preliminary experiments that 2 U of insulin did not increase plasma insulin levels. Blood was sampled exactly 14 d after insertion.

In Experiment 3, after an initial blood sample from the tail, 10 nondiabetic BB rats were infused intravenously via a catheter in the jugular vein, inserted under pentobarbitone anesthesia, with a glucose 30% solution for 96 h, at which time another blood sample was taken under anesthesia.

In Experiment 4, after an initial blood sample from the tail, 14 diabetic rats were ADX by two small costolumbar incisions under pentobarbitone anesthesia. During the first 24 h, the rats received a 5% glucose- and 0.9% NaCl-containing solution for drinking, and a 0.9% NaCl solution thereafter. Seven of the ADX rats were treated with sc injections of corticosterone (Merck, Darmstadt, Germany; 15 mg/d, dissolved in propyleneglycol). We used about the same dose (50 mg/kg) as Unterman et al. *(21)*; the daily dose was divided into several injections during the working day, and the last injection was given some hours before blood sampling, which was exactly 96 h after ADX.

Assays

Plasma glucose was measured with a glucose-oxidase method (Beckman Glucose Analyzer, Fullerton, CA). Insulin was measured by radioimmunoassay (RIA) with rat insulin as standard *(8)*. The plasma concentrations of DBP and of CBG were determined by single radial immunodiffusion using rat standards *(36,37)*. OC was measured by RIA with rat OC as standard; the intra- and interassay coefficients of variation were 5.9 and 5.2%, respectively *(38)*.

Corticosterone was measured by both competitive protein binding assay and RIA. In the RIA, a commercially available polyclonal rabbit antiserum against corticosterone (UCB, batch 250485) and radiolabel ([1,2,6,7-^{3}H] corticosterone) with an SA of 1.0 mCi/mL (Amersham, Buckinghamshire, UK) were used. Corticosterone standards, controls (corticosteroid-free plasma), and plasma samples in duplicate were first extracted with CH_2Cl_2. Assay tubes containing 100 µL extract, 500 µL radiolabel (at 1 µCi/35 mL), and 100 µL antiserum (diluted 1:15,000) were incubated for 30 min at 37°C, and then for 16 h at 4°C. Free and bound corticosterone were separated by the addition of 500 µL dextran-coated charcoal, incubated for 30 min at 4°C, centrifuged (10 min at 4,000*g*), and the radioactivity of 500 µL of the supernatant was counted.

IGF-I was measured by radioimmunoassay as described *(8)*, but in order to maximally remove the IGFBPs, we used Econo-Pac columns. All the following procedures were done at 4°C. Forty grams of Bio-Gel P30 (Bio-Rad, Richmond, CA), with a particle size ranging from 90–180 µm (mol wt fractionation range: 2500–40,000 Dalton), were equilibrated overnight with 720 mL buffer containing 1 mol/L acetic acid and 0.025 mol/L NaCl, and degassed for 10 min. After at least 90% of the particles in the gel had settled, the supernatant was decanted, and the gel slurry was poured into Econo-Pac columns (Bio-Rad) up to 9.5 cm. Samples were prepared by adding 16 µL of formic acid to 400 µL of plasma sample in an Eppendorf tube, vortexing, and incubating overnight. After centrifugation, 200 µL of the supernatant were applied to the column. The IGFBPs were eluted by adding 11 of mL elution buffer (with 1 mol/L acetic acid and 0.025 mol/L NaCl). The IGFs were then eluted into another tube by adding 8 mL elution buffer. Five hundred microliters of this eluate were dried by Speed Vac centrifugation, dissolved into 500 µL neutral assay buffer, of which 50 µL in duplicate were used for the RIA. Figure 7 shows that the "IGF eluate" (fractions 12-19) contained most of the radiolabeled IGF-I. Addition of 100, 200 and

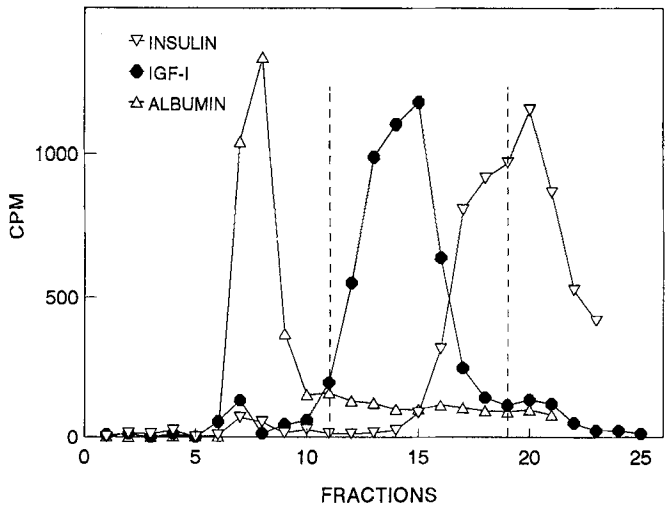

Fig. 7. Separation of IGF-I, insulin, and albumin using Econo-Pac columns (Bio-Rad, Richmond, CA). ^{125}I-albumin, ^{125}I-insulin, or ^{125}I-IGF-I were added to acidified serum (25 serum: 1 formic acid, pH 2.2), applied to the Econo-Pac columns, and eluted with buffer containing 1 mol/L acetic acid and 0.025 mol/L NaCl (pH 2.2). Fractions 1–11 contained most of ^{125}I-albumin, whereas ^{125}I-IGF-I was eluted in fractions 12–19.

400 ng/mL of IGF-I to serum showed a recovery of 95 ± 6% (mean ± SD; n = 4), and in the dilution curve, the mean percentage of found over expected IGF-I concentrations was 100 ± 6% (mean ± SD; n = 5). The intra- and interassay coefficients of variation were 4.6% (n = 5) and 11.3% (n = 6), respectively. A comparable method for removal of IGFBPs has been described by Mohan and Baylink *(39)*, and was found to have a correlation of more than 90% with IGF-I concentrations obtained after acid gel filtration with Sephadex G-75 columns, the gold standard for removal of IGFBPs, but impractical because it is time-consuming.

Western ligand blotting was performed as described by Hossenlopp et al. *(40)*, with minor modifications. Rat plasma samples (diluted 1:8) were subjected to 12.5% homogenous SDS-PAGE under nonreducing conditions (max 250 V, 4–5 h). The electrophoresed proteins were transferred onto nitrocellulose sheets in Tris-glycine-methanol buffer, washed in 0.1% Tween saline, and incubated overnight with [^{125}I]-IGF-I (600,000 cpm/mL PBS + 1% BSA buffer), washed again, and dried. Blots were autoradiographed for 4–5 d at –70% with Hyperfilm TM-MP (Amersham, UK), and then scanned with an Ultroscan XL Laser Densitometer (Pharmacia, Uppsala, Sweden). A standard plasma sample (from a control rat, d 0) was included in each run, and the results were expressed as the ratio compared with this sample, which was standardized as 1.00 arbitrary units.

Data Analysis

Results were expressed as means ± SEM, and were analyzed using a software program (NCSS, Kaysville, UT). Paired t-tests were used to compare values from diabetic rats with the respective control littermates (Experiment 1), or the pre- with posttreatment values (Experiments 3 and 4). Since Experiment 2 involved multiple groups with a dissimilar number of data, statistical analysis was carried out by one-way ANOVA to detect overall differences, and if $p < 0.05$, was followed by Tukey's multiple comparison test; significant differences between groups were then assessed by unpaired t-tests.

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