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Physiology

Epidermal growth factor deficiency associated with diabetes mellitus

(C57BL/KsJ *db/db* mice/insulin/streptozotocin/submandibular gland/prepro-epidermal growth factor mRNA)

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ABSTRACT **The production of epidermal growth factor (EGF) in the submandibular gland and its circulating level were studied in diabetic mice. In genetically diabetic (C57BL/KsJ *db/db*) mice, EGF concentrations in the submandibular gland and plasma were reduced to 13% and 30% of the control levels, respectively. In streptozotocin-treated diabetic mice, they were reduced to 18% and 20% of controls, respectively, 5 weeks after the drug injection. Furthermore, levels of submandibular prepro-EGF mRNA in these diabetic mice were decreased almost in parallel with the glandular EGF concentrations, while there was no change in the levels of submandibular β-actin mRNA and kidney prepro-EGF mRNA. In addition, histological examination of the submandibular glands indicated that the size of the granular convoluted tubules, which produce EGF, was substantially reduced in the diabetic mice. Insulin administration to streptozotocin-treated mice almost completely reversed the decrease in EGF content in the submandibular gland, substantially elevated the level of the glandular prepro-EGF mRNA and plasma EGF concentration, and increased the size of the granular convoluted tubules in the gland. These results indicate that EGF deficiency occurs in diabetes mellitus and that insulin may be important in maintaining the normal level of EGF in the submandibular gland and plasma.**

Epidermal growth factor (EGF) exerts a variety of biological activities on various mammalian cells (1, 2). In mice, the submandibular gland is the major site for EGF production and the major source of circulating EGF (3–5). Studies on experimentally induced EGF-deficient mice have suggested that EGF is important in many physiological processes—for example, spermatogenesis (3), completion of normal pregnancy (4), mammary gland development (6, 7), and wound healing (8–10). Thus, the possibility exists that EGF deficiency contributes to the pathology of disease states. Diabetes mellitus is of interest in this respect, since some of the pathology accompanying the disease includes disorders ordinarily associated with EGF deficiency (11, 12). We therefore examined the production of EGF by the submandibular gland and its plasma level in two different kinds of diabetic mouse models—namely, genetically diabetic (*db/db*) mice (12) and mice with streptozotocin-induced diabetes (13). The genetically diabetic (*db/db*) mice show hyperglycemia, progressive weight gain, and extreme insulin resistance associated with increased insulin secretion during the period between 10 days and 5–6 month of age (12). In contrast, streptozotocin produces pancreatic insulitis leading to insulin deficiency with progression to diabetes mellitus (13). Our results indicate that in these diabetic mice, the levels of EGF and its mRNA in the submandibular gland as well as circulating EGF are greatly reduced.

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MATERIALS AND METHODS

Diabetic Mice. Male C57BL/KsJ diabetic (*db/db*) mice were purchased from The Jackson Laboratory. Nondiabetic C57BL/KsJ homozygotes (+/+) were used as control mice. Mice with streptozotocin-induced diabetes were produced by injecting streptozotocin (50 μg per g of body weight) i.p. to C57BL/KsJ +/+ mice for 5 consecutive days (13). The mice were sacrificed at 1, 3, or 5 weeks after the first injection of streptozotocin. Some of the mice with streptozotocin-induced diabetes received a daily s.c. injection of 1 unit of human NPH insulin (Eli Lilly) at 6 PM. The insulin administration was started 1 week after the first injection of streptozotocin and continued for 2 weeks until the mice were sacrificed. All mice were 13–15 weeks old at the time of sacrifice. Plasma glucose concentrations were determined with a glucose diagnostic kit (Sigma).

EGF Radioimmunosassy. The extracts of the submandibular glands were prepared as described (5). EGF concentrations in the submandibular gland extracts and plasma were determined by radioimmunoassay using a liquid-phase double antibody method (5).

Western Blotting. Submandibular gland extracts were mixed with 1/10th vol of 10× SDS sample buffer (1× SDS = 10 mM Tris·HCl/1% SDS/2% 2-mercaptoethanol/0.04% bromphenol blue, pH 6.8) and heated at 100°C for 5 min. After electrophoresis in an 8–25% SDS/polyacrylamide gel gradient, the proteins were transferred by diffusion to nitrocellulose membranes at 70°C. The antigen–antibody reactions were done with rabbit anti-mouse EGF antiserum (Collaborative Research) and were detected by goat anti-rabbit IgG colloidal gold conjugate (Bio-Rad) (14). The blots were amplified with silver lactate (15).

Extraction and Analysis of RNA. The 5′ end *Pst* I fragment of the plasmid pmEGF-26F12 (16), constructed from a 960-base-pair fragment of mouse prepro-EGF cDNA, was labeled with [α-^{32}P]dCTP by an oligonucleotide labeling method (17). Radiolabeled RNA probe for mouse β-actin mRNA (18) was prepared as described (19).

Total cellular RNA was isolated from the submandibular glands and the kidneys according to Chirgwin *et al.* (20). For Northern blot analysis, 10 μg of RNA was denatured in formamide/formaldehyde solution and electrophoresed in 1% agarose/2% formaldehyde gels as described (21). The gels were transferred to nitrocellulose filters. For dot blot hybridization, up to 5 μg of RNA sample was loaded onto nitrocellulose filters. The filters were baked, prehybridized, and hybridized to radiolabeled probes for 24 hr at 42°C (prepro-EGF mRNA) or at 55°C (β-actin mRNA) as described (19). The blots were washed first at room temperature in 2× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0)/0.1% SDS, and then at 50°C (prepro-EGF mRNA) or 65°C (β-actin mRNA) in 0.1× SSC/0.1% SDS. The washed

Abbreviation: EGF, epidermal growth factor.

filters were autoradiographed with Kodak XAR-2 film with an intensifying screen.

Histology. The submandibular glands were removed, fixed in 10% buffered formalin (pH 7.0), and embedded in paraffin wax. Tissue sections were prepared (5 μm thick) and stained with hematoxylin and eosin.

RESULTS

Genetically diabetic C57BL/KsJ *db/db* male mice exhibited marked obesity [mean body weight, 43.5 ± 1.4 g (SE) (n = 13) vs. 26.4 ± 0.4 g (SE) (n = 13) in controls] and hyperglycemia. The mean plasma glucose concentrations of diabetic and control mice were 650 ± 15 mg (SE) per 100 ml and 180 ± 16 mg (SE) per 100 ml, respectively. The concentration of EGF in the submandibular glands of the diabetic (*db/db*) mice was reduced to 13% of the control level (Fig. 1). Western blots of extracts of the submandibular glands from both the control and the diabetic (*db/db*) mice showed the major band corresponding to authentic mature ≈6-kDa EGF and indicated that the amount of ≈6-kDa EGF in the tissue extracts of the diabetic (*db/db*) mice was much less than that of the control (Fig. 2). The level of EGF in plasma in the diabetic animals was reduced by 70% when compared with control (Fig. 1).

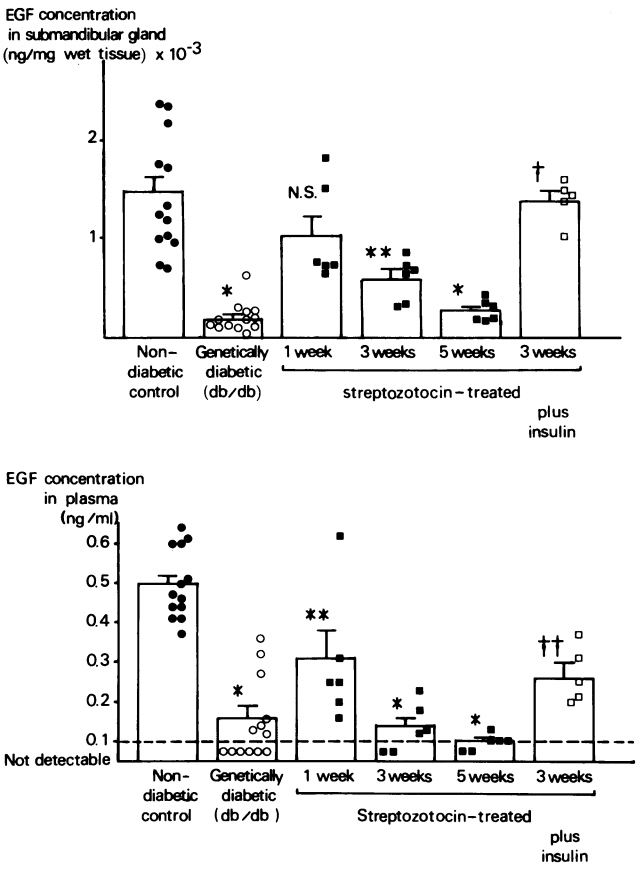

FIG. 1. EGF concentration in submandibular glands (*Upper*) and plasma (*Lower*) of control, genetically diabetic (*db/db*), and streptozotocin-treated diabetic mice. Some of the mice with streptozotocin-induced diabetes received a daily administration of insulin. The insulin injection was started 1 week after the first injection of streptozotocin and continued for 2 weeks until the mice were sacrificed. The weights of the submandibular glands were 29 ± 1 mg (SE) in the genetically diabetic (*db/db*) mice; 61 ± 1 mg (SE) in controls; and 49 ± 3 mg (SE), 44 ± 2 mg (SE), and 33 ± 2 mg (SE) in the streptozotocin-treated mice 1, 3, and 5 weeks after the injection. Each circle and square represents the value of an individual animal. Columns show means ± SE. When the values of plasma EGF were below the sensitivity of the assay (0.1 ng/ml), they were assigned to 0.1 ng/ml for the calculations. *, $P < 0.001$ and **, $P < 0.01$, significant differences when compared with control mice. †, $P < 0.001$ and ††, $P < 0.05$, significant differences when compared with streptozotocin-treated mice at 3 weeks. N.S., not significant (Student's t test).

Multiple injections of streptozotocin into nondiabetic male C57BL/KsJ mice produced a gradual increase in plasma glucose concentration; the mean plasma glucose levels were 180 ± 18 mg (SE) per 100 ml (n = 13), 347 ± 38 mg (SE) per 100 ml (n = 6), 521 ± 27 mg (SE) per 100 ml (n = 6), and 502 ± 23 mg (SE) per 100 ml (n = 6) at 0, 1, 3, and 5 weeks after the injection, respectively. The EGF concentrations in the submandibular glands decreased in a time-dependent fashion: the levels were 70%, 40%, and 18% that of control mice at 1, 3, and 5 weeks, respectively, after the drug injection (Fig. 1). Streptozotocin treatment also progressively reduced the plasma concentration of EGF so that 5 weeks after the injection it was reduced to 20% of control levels (Fig. 1). Daily administration of insulin to streptozotocin-treated animals restored the glandular EGF concentration to almost the control level; the plasma level of EGF was also increased although it did not reach the level in control animals (Fig. 1). Similar attempts to increase the EGF concentration in the submandibular gland and plasma of the genetically diabetic (*db/db*) mice by insulin treatment were not made because these animals are extremely resistant to insulin (12).

EGF is derived from a precursor molecule, prepro-EGF, of 1217 amino acids (16, 22). Large amounts of prepro-EGF mRNAs are present in the submandibular gland and the kidney (23). In both genetically diabetic (*db/db*) and streptozotocin-induced diabetic mice, the levels of 4.7-kilobase (kb) prepro-EGF mRNA in the submandibular gland were much lower than those of control mice (Fig. 3). Densitometric scan of the dot blots showed that in the genetically diabetic (*db/db*) mice the relative abundance of the mRNA was 7% of controls. In streptozotocin-treated animals the level of prepro-EGF mRNA declined progressively after the drug injection to 15% of controls 5 weeks after the injection. However, daily administration of insulin to the streptozotocin-treated mice for 2 weeks significantly ($P < 0.01$) increased the prepro-EGF mRNA level (Fig. 4). In contrast, levels of 2.0-kb mRNA for structural protein β-actin in the gland did not appear to be changed in those diabetic mice (Fig. 3). In addition, levels of prepro-EGF mRNA in the kidneys of diabetic mice were not significantly different from those in the control (Fig. 3).

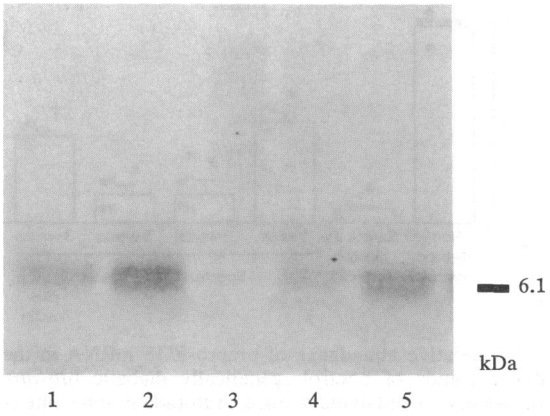

FIG. 2. Western blot analysis of EGF in the submandibular gland of control and genetically diabetic (*db/db*) mice. Lanes 1 and 2, control; 3 and 4, *db/db*; 5, standard EGF (3 ng). The protein concentration of tissue extract was 3 μg (lanes 1 and 3) or 5 μg (lanes 2 and 4) in 1 μl per lane.

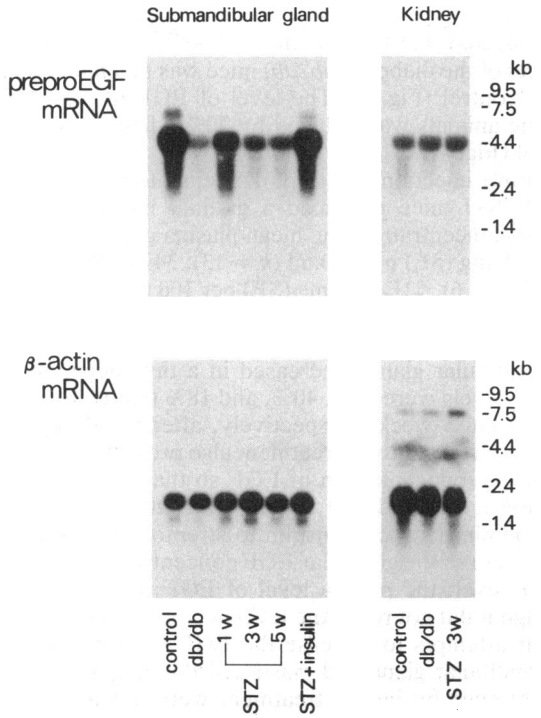

FIG. 3. Northern blot analysis of prepro-EGF mRNA (*Upper*) and β-actin mRNA (*Lower*) in the submandibular glands and kidneys. The tissues were obtained from control, genetically diabetic (*db/db*), and streptozotocin-induced diabetic (STZ) mice. Some of the streptozotocin-treated mice received insulin as described in the legend to Fig. 1. The RNA from the submandibular gland and kidney was obtained from the same animal. The data shown are representatives of at least three experiments. Sizes of RNA markers (BRL) are indicated on the right.

Prepro-EGF mRNA and EGF are produced in the granular convoluted tubules of the submandibular glands (23, 24). Histological studies showed that the size of the granular convoluted tubules in the glands of genetically diabetic (*db/db*) mice (Fig. 5*B*) was about one-third that of the controls (Fig. 5*A*). In mice with streptozotocin-induced diabetes (Fig. 5*C*), the size of the tubules was less than half that of the controls. In contrast, when insulin was administered to the streptozotocin-treated mice, their tubular cells became larger, resembling those from the control mice (Fig. 5*D*). Thus, insulin replacement was effective in restoring normal morphology of the gland in the streptozotocin-treated diabetic mice. There was no obvious inflammatory process in the glands of diabetic mice to account for the altered levels of prepro-EGF mRNA and EGF.

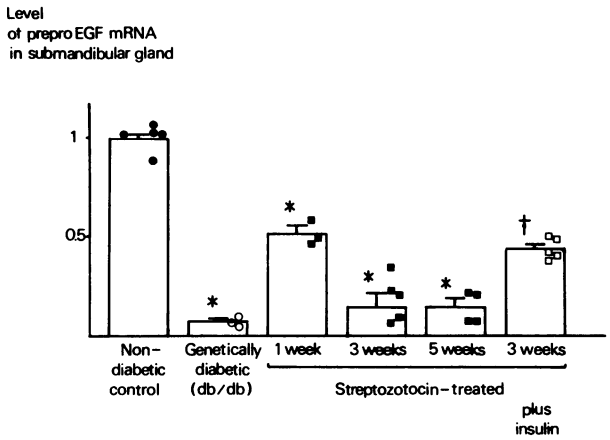

FIG. 4. Relative abundance of prepro-EGF mRNA in the submandibular glands of control, genetically diabetic (*db/db*), and streptozotocin-induced diabetic mice. Autoradiographic intensity of dot blot hybridization was expressed as the value normalized to that in the submandibular glands of control mice. Each circle and square represents the value of an individual animal. Columns represent means ± SE. *, $P < 0.001$, significant differences when compared with control mice. †, $P < 0.01$, significant differences when compared with streptozotocin-treated mice at 3 weeks (Student's *t* test).

DISCUSSION

The present study showed that in two kinds of diabetic mice the levels of prepro-EGF mRNA and EGF in the submandibular gland were greatly reduced. Histological examinations of the submandibular glands indicated that the granular convoluted tubules in diabetic mice were much smaller in diameter. Since prepro-EGF mRNA and EGF are produced in these tubule cells (23, 24), such findings are consistent with the biochemical difference between the glands of diabetic and control mice. Thus, EGF synthesis in the glands of diabetic mice appeared to be impaired mainly at pretranslational levels involving transcription and/or mRNA turnover. On the other hand, structural protein β-actin mRNA levels were not changed in the submandibular glands of diabetic animals, suggesting that the decrease in prepro-EGF mRNA in diabetes is not caused by a nonspecific decrease in cellular RNA levels in the submandibular gland. In addition, no apparent difference in the level of kidney prepro-EGF mRNA was found between the diabetic and control mice. Thus, the decrease in EGF production in diabetes is a specific phenomenon in the submandibular gland.

Our studies also indicated that administration of insulin to the streptozotocin-treated animals corrected the defect in EGF production by increasing the levels of prepro-EGF mRNA and EGF in the gland. Histological findings also support these findings. These results are consistent with the view that the changes in EGF production are not attributable to the toxic effects of streptozotocin or to obesity but reflect a general phenomenon in diabetes mellitus. In addition, our preliminary studies showed that the glandular EGF content in female diabetic (*db/db*) mice was also greatly reduced—i.e., to 13% that of female controls (unpublished data) and thus indicated that the EGF deficits in diabetic (*db/db*) mice are common to both sexes. These results suggest that such deficits are independent of androgen actions, although the levels of prepro-EGF mRNA and EGF in mouse submandibular gland are stimulated by androgens (25–27).

It remains to be determined whether insulin acts on the gland directly or indirectly. Since insulin receptors are present in the submandibular glands (28), it is possible that insulin exerts its effect directly on the tubule cells in maintaining the normal level of prepro-EGF mRNA and EGF production. However, in diabetic animals there are a number of metabolic abnormalities (29), including hyperglycemia and alterations in the levels of hormones and enzymes, which may be responsible for the decrease in EGF production. Thus, the possibility exists that the decreased synthesis of EGF in diabetic mice is due to complex indirect consequences of insulin deficiency or insulin resistance.

Relatively large amounts of 4.7-kb prepro-EGF mRNA are also found in the kidney (23, 26). However, in contrast to the submandibular gland, its primary translated product, prepro-EGF, is apparently not processed to mature ≈6-kDa EGF in this tissue. Our data showed that prepro-EGF mRNA in the kidney of diabetic mice was at normal levels, indicating that the regulation of EGF gene expression differs in the submandibular gland and the kidney (26). At present, the physiological role of kidney EGF is not known.

The decreased plasma EGF concentration in diabetic mice may be attributable to decreased synthesis of EGF by the

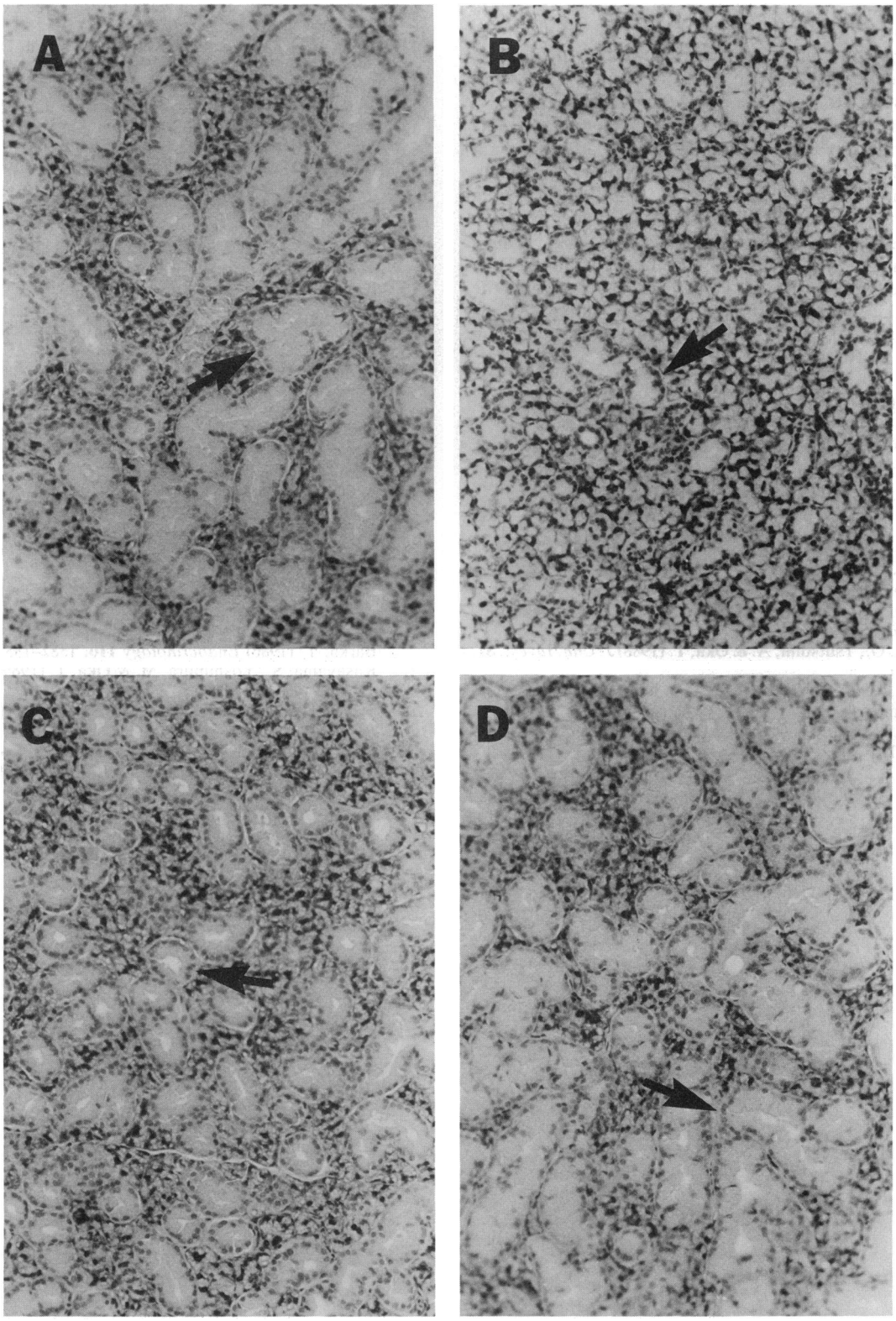

FIG. 5. Histology of submandibular glands from control mice (*A*), genetically diabetic (*db/db*) mice (*B*), and streptozotocin-induced diabetic mice 3 weeks after drug injection without (*C*) or with (*D*) daily administration of insulin for 2 weeks. Sections (5 μm) were stained with hematoxylin and eosin. Arrows indicate granular convoluted tubules. The sizes of the granular convuluted tubules (longer axis × shorter axis) of the control, the genetically diabetic (*db/db*), the streptozotocin-induced diabetic, and the insulin-treated mice were estimated to be 7100 ± 900, 2000 ± 200*, 2700 ± 400*, and 6800 ± 500† μm^2, respectively (means ± SE; $n = 10$). *, $P < 0.001$, significant differences when compared with control mice. †, $P < 0.001$, significant differences when compared with streptozotocin-treated mice without insulin treatment (Student's *t* test). (×180.)

submandibular glands. It has been shown that removal of the submandibular glands reduces the plasma EGF level (3–6) and that the release of EGF from submandibular glands into the circulation can be provoked by pharmacological stimuli (25, 30). However, since the physiological mechanisms of EGF secretion into the circulation remain unclear, it is

possible that the process of EGF secretion is also defective in diabetic mice. In fact, it is noteworthy that insulin treatment of streptozotocin-induced diabetic mice elevated the plasma EGF concentration only partially, while it normalized the EGF content stored in the gland almost completely.

Finally, the present study suggests an intriguing connection between insulin and EGF that operates via the submandibular gland in the following way: insulin directly or indirectly facilitates EGF production in the gland, and EGF, in turn, exerts its effect on target cells alone or in combination with insulin to help maintain homeostasis. Recently, we have shown that insulin is also important for the regulation of EGF receptor gene expression (19). Thus, in diabetes mellitus the interplay of the two polypeptides may be disrupted, leading to manifestation of pathological complications in certain tissues.

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