

SALIVARY GLAND STRUCTURE AND FUNCTION IN EXPERIMENTAL DIABETES MELLITUS

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SUMMARY

• *While salivary secretion is not initiated by circulating hormones, there are significant endocrine influences on the development, structure and function of salivary glands. Experimental animal models of diabetes mellitus have been used to study all aspects of diabetic pathophysiology. There is now a considerable body of evidence demonstrating that the effects of diabetes on rodent salivary glands are related as much to the indirect consequences of insulin insufficiency on the circulating levels of other hormones, and autonomic nerve function, as to the direct actions of insulin. In addition to their exocrine functions, salivary glands also play an endocrine role. Rodent submandibular glands, in particular, are a rich source of biologically active polypeptides, which are synthesized in the granular ducts. Although many of these polypeptides are released into both the blood and the saliva, their physiological functions have yet to be fully explored. Nevertheless, the disruption of submandibular gland endocrine function has been postulated to contribute to the pathology observed in diabetic animals. (BiomedRev 1998; 9: 107-119)*

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INTRODUCTION

• The function of most glands in the digestive tract is dependent, at least in part, on hormonal stimulation, but salivary gland function has been regarded as primarily, if not exclusively, under the control of autonomic nerves. Nonetheless, while salivary secretion is not initiated by circulating hormones, there are significant endocrine influences on the development, structure and function of salivary glands. Moreover, salivary glands have been postulated to have endocrine functions of their own. Rodent submandibular glands (SMG), in particular, synthesize and release into the blood a variety of biologically active polypeptides for purposes that remain to be fully elucidated.

• **Diabetes mellitus**

The term diabetes mellitus refers to a group of heterogeneous disorders that share the common feature of glucose intolerance, and the key defect that leads to the onset of insulin-dependent diabetes mellitus (IDDM, type I) is an autoimmune destruction of β cells in the pancreas and, as a consequence, a loss of insulin producing capacity. In contrast, non-insulin-dependent diabetes mellitus (NIDDM, type II), which occurs most commonly in adults, is characterized by a derangement of insulin secretion and the development of insulin resistance. The binding of insulin to its receptor rapidly directs metabolism toward anabolic and away from catabolic pathways, thereby promoting glucose transport and carbohydrate metabolism, fatty acid synthesis, amino acid transport, proteins synthesis,

and mRNA synthesis. Experimental animal models of human diabetes mellitus have been used to study all aspects of diabetic pathobiology, but the specific effects of diabetes in any given tissue, including the salivary glands, are related as much to the indirect consequences of insulin insufficiency on blood chemistry, circulating levels of other hormones, and autonomic nerve function, as to the direct actions of insulin.

- **Animal models of diabetes mellitus**

The diabetogenic effects of alloxan were first reported in 1943 by Dunn and colleagues, and its cytotoxic effects result from the intracellular generation of reactive compounds such as superoxide anion and hydroxyl radicals (1). At least in the rat, alloxan also displays a significant degree of extrapancreatic, hepatic and renal, toxicity, and because of this streptozotocin has largely replaced alloxan as the diabetogenic agent of choice for this species (2). Both alloxan- and streptozotocin-diabetic animals exhibit polydipsia, polyuria and glycosuria, but unlike human diabetes mellitus the hyperglycaemia is seldom accompanied by ketoacidosis. As a result, alloxan- and streptozotocin-treated animals can usually be maintained in a stable diabetic state indefinitely. A spontaneous animal model of IDDM, the BioBreeding (BB) Laboratories Wistar rat, has also been used to study the dynamic physiological changes that occur in diabetes (3). These animals develop a syndrome that closely resembles the human form of the disease, both in time of onset and severity. Other spontaneous models of diabetes include the genetically diabetic (*db/db*) and the non-obese diabetic (NOD) mice. The *db/db* (C57BL/KsJdb) mouse is a model of NIDDM, and hyperglycaemia prevails in company with normal or sometimes even elevated circulating levels of insulin (4). The NOD mouse, on the other hand, appears to be more suitable for the study of autoimmune salivary gland diseases, such as Sjögren's syndrome (5).

SALIVARY GLAND STRUCTURE AND EXOCRINE FUNCTION IN DIABETES MELLITUS

- The gross morphological effects of diabetes on the salivary glands have been well documented. Initial reports demonstrated that the induction of alloxan diabetes led to a reduction in parotid gland and SMG weights (6,7). Virtually all subsequent studies confirmed these findings (8-21), and with only minor exceptions the SMG was more susceptible than the parotid gland. Insulin treatment reversed the effects of diabetes, and thus diabetes and insulin are generally considered to have parallel effects on salivary gland and body growth. The notable exception was the rat sublingual gland, which was relatively unaffected (14,20). Liu and Lin (6,7) quickly realized, however, that the effects of diabetes on SMG growth were not due to insulin insufficiency alone. The combination of insulin and growth hormone, for example, was more effective than either hormone

alone in reversing the effects of diabetes on the rat SMG. Moreover, diabetes resulted in a decrease in the weights of several endocrine glands, including the thyroid, the adrenal and the pituitary, suggesting that the effects of diabetes on salivary glands might be mediated indirectly through a decrease in the circulating levels of hormones other than insulin.

- **Parotid and submandibular acinar cell structure and function**

Early morphological studies indicated that parotid and SMG acinar cell size was reduced in alloxan-diabetic rats (6). Later studies were unable to confirm such changes in short-term, 3-12 weeks duration, streptozotocin-diabetes, but a reduction in SMG acinar cell size was observed in long-term, 4-6 months, streptozotocin-diabetic rats (17). Electron microscopy revealed a variety of pathological changes, including a pooling of secretory material and an increase in the number of autophagic vacuoles in the rat SMG (17), and the presence of membrane bound cytoplasmic "crystalloids", which appeared to be lysosomal in nature, in the parotid gland (8,18). However, the most striking morphological abnormality found was the accumulation of lipid droplets in the basal regions of serous acinar cells (Fig. 1,2). The appearance of lipid in the parenchymal cells was rapid, beginning within 24 hours after the induction of diabetes, and it persisted indefinitely in the absence of insulin treatment. Little or no lipid was observed in the mucous acinar cells of the sublingual glands, and except in a few intercalated ducts, none was found in ductal cells. While lipid droplets were observed in all three major salivary glands of the rat, its accumulation in the parotid gland was particularly impressive (Fig. 2). On average, 8-10 % of the acinar cell volume was occupied by lipid, but in some instances the lipid displaced most of the intracellular organelles, causing the acinar cells to superficially resemble adipocytes. In frozen sections, the lipid stained with Oil Red O and calcium lipase suggesting that it was comprised of neutral lipids, primarily triglycerides (21). Although the increased lipid could not be attributed to any one class, there were changes in fatty acid composition; C18:0 (stearic acid) and C: 18:2co6 (linoleic acid) increased while C18:1 co9 (oleic acid) and C20:4co6 (arachidonic acid) decreased (22). Why the acinar cells should accumulate lipid in diabetes is unknown, but three possible explanations present themselves: (i) the lipid may serve as a potential source of energy in the absence of glucose transport as it does in cardiac and skeletal muscle (23,24), (ii) lipid accumulation may occur consequent to nonspecific endocytosis of excess circulating lipid (25); serum triglyceride levels are elevated in diabetic rats (18), and (iii) lipid storage might be enhanced due to a decrease in its utilization for the packaging of secretory proteins. Insulin appears to directly stimulate acinar cell secretory protein synthesis in both the parotid and SMG. Parotid amylase levels were reduced in diabetes and insulin rapidly enhanced amylase and amylase mRNA synthesis (8-11). Similarly, the

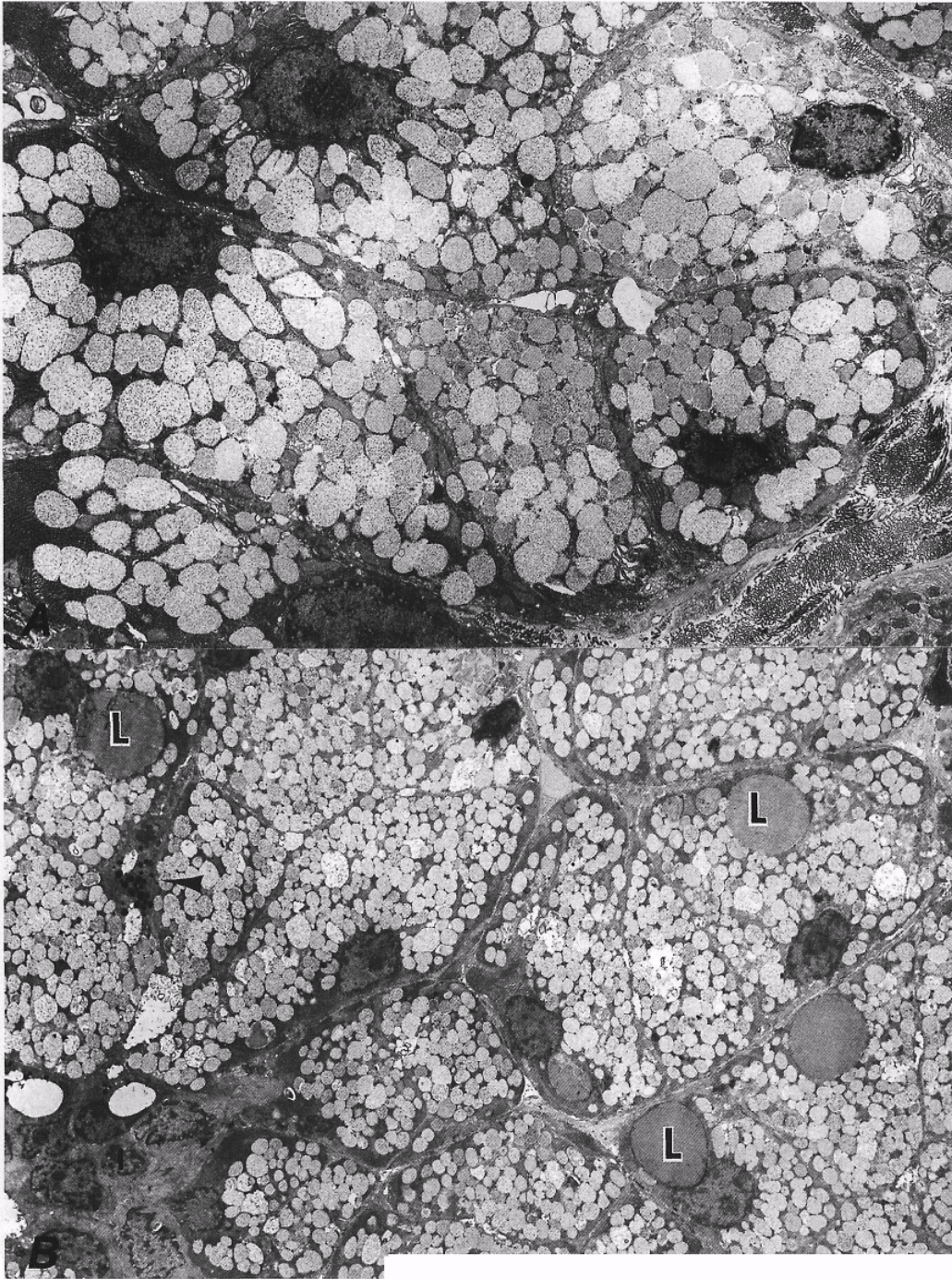


Figure 1. Electron micrograph of the submandibular gland from a control rat. The acinar cells are relatively uniform in size, and packed with electron-lucent secretory granules (A). Electron micrograph of the submandibular gland from 3-week streptozotocin-diabetic rat. Lipid droplets (L) are present in the acinar cells, but not in intercalated duct cells. Note the small serous-like granules in the cells of the acinar-intercalated duct interface (arrow) (B). $\times 4\,400$ (A), $\times 2\,090$ (B). From Ref 13.

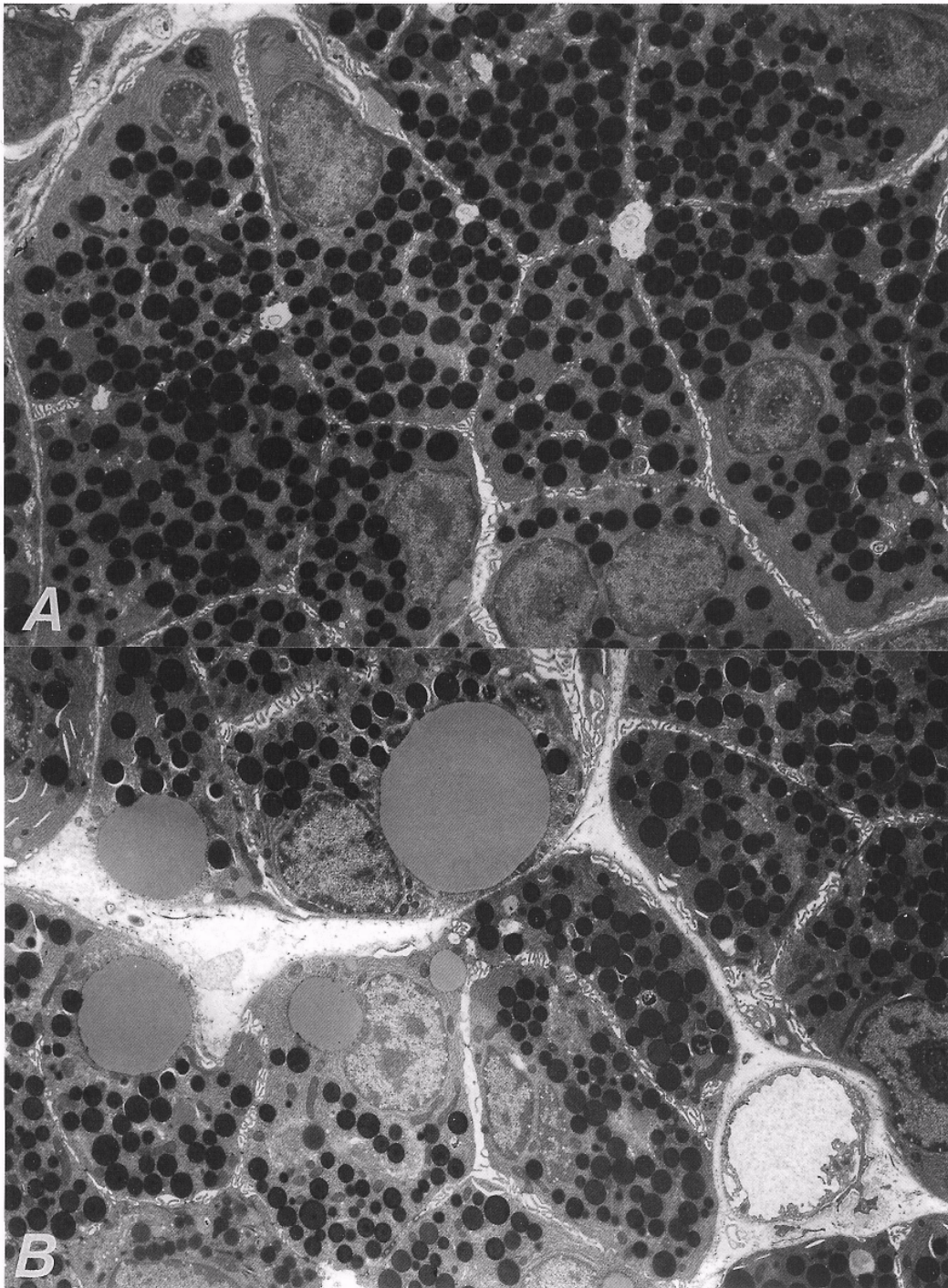


Figure 2. Electron micrograph of the parotid gland from a control rat. The acinar cells are relatively uniform in size, and are packed with electron-dense secretory granules (A). Electron micrograph of the parotid gland from a 3-week streptozotocin-diabetic rat (B). Lipid droplets of varying size are present in the basal portions of the acinar cells, x 3 500 (A, B). From Ref69.

activity of acinar cell-derived peroxidase in the SMG was decreased in diabetic rats (13). Insulin treatment *in vivo* led to an increase in peroxidase activity and a stimulation of amino acid incorporation into proteins. *In vitro* studies confirmed that insulin rapidly stimulates protein synthesis in the rat SMG (26). The effects of insulin, however, were more specific than a general upregulation of protein synthesis as not all secretory proteins were equally affected by diabetes and insulin (8). Insulin also stimulated the incorporation of glucose into SMG and sublingual gland glycoproteins. The depression of glucose incorporation was correlated with a decrease in the activities of several enzymes involved in the biosynthesis of hexosamines and sialic acid (12,27-30). Conversely, an enzyme involved in the degradation of glycoproteins and glycosaminoglycans, N-acetyl-(3-D-glucosaminidase, was increased in streptozotocin-diabetes, and its activity was depressed by insulin (31). Differences in carbohydrate metabolism were also revealed histochemically using glycoconjugate staining (20).

D Granular duct structure and gene expression

Rodent SMG are characterized by a highly specialized portion of the ductal system, the granular convoluted ducts (Fig. 3), whose secretory cells synthesize a variety of biologically active polypeptides, including nerve growth factor (NGF), renin and erythropoietin in the mouse, and epithelial growth factor (EGF) and kallikreins in the mouse and rat (32,33). While the physiological functions of many granular duct-derived peptides have yet to be explored fully, the granular ducts themselves are an excellent system to study hormonal regulation of gene expression. The effects of diabetes on the granular ducts were first noted by Parhon *et al* (34), and then independently by Liu and Lin (6,7). Granular duct diameters were reduced in alloxan diabetic rats, and a similar decrease in granular duct size was observed in male *db/db* mice (35). In female *db/db* mice the granular ducts actually became more prominent. As the effects of diabetes on granular duct structure were generally ascribed to an impairment of the hypothalamic-pituitary axis (see below), masculinization of the SMG in female *db/db* mice was thought to result from the effects of adrenal androgens, or from a defect in the conversion of ovarian androgens to estradiol. Despite the reduction in size, the overall granular duct cell morphology at the electron microscopical level (Fig. 3 B) was relatively unaffected in diabetic animals (17,18). Diabetes also had a profound effect on the protein synthesis by mouse granular duct cells; the levels of EGF and NGF decreased by greater than 50% in streptozotocin-diabetic and *db/db* mice (36-38). Protease activities, including those of glandular kallikrein and tonin in diabetic rats (Fig. 4) and renin in mice, were also decreased (16,32,39-41). Insulin treatment led to a complete recovery of NGF, EGF and tissue kallikrein content in the mouse SMG. However, unlike acinar cell secretory proteins, the recovery of protein levels in the granular ducts required one week or more of insulin treatment. In the ca-

se of some kallikrein-like proteases and tonin in the rat SMG, insulin appeared to have little, if any, effect over this time course. The induction of diabetes causes a reduction in the plasma concentrations of testosterone, thyroxine and growth hormone (42-44), and the recovery of normal plasma levels of these hormones occurs only with extended insulin treatment. Taken together these data support the hypothesis that the maintenance of granular duct gene expression is not directly dependent on insulin, and a brief review of the multihormonal regulation of granular duct structure and function is therefore warranted.

• Multihormonal influences on granular duct gene expression

Differences in granular duct structure between males and females have been described for a number of different species (32). The initial observations of a sexual dimorphism in the mouse SMG were reported independently by Lacassagne (45) and Fe-kete (46). The granular ducts were more prominent in male mice than in females, and this difference was dependent on the presence of androgen. However, androgens were not the only endocrine influences on the granular ducts by demonstrating that in male mice hypophysectomy had a greater effect than did castration (47). These results were made clear when it was shown that the development and maintenance of the granular ducts is dependent on the interaction of sexual, thyroid and adrenocorticoid hormones (48). Over the next three decades, a variety of experimental approaches confirmed and extended these findings, and the reader is encouraged to see the reviews published (32,49).

Studies conducted in the late 1940's by Junqueira *et al* (50) and later by Sreebny and Meyer (51) correlated the differences in granular duct histology and cytochemistry with the levels of protease activity in glandular extracts. Protease activity in female mice was significantly less than that in males, and in prepubertal mice enzymatic activity was very low and did not differ between males and females. In addition, protease content decreased in male SMG following castration, and increased in normal female and castrated male mice upon the administration of testosterone. These early studies established that androgens influence the levels of protease activity in the mouse SMG. Numerous studies have since demonstrated the effects of pituitary-dependent hormones on SMG proteases (32,52,53). The sexual dichotomy between male and female rats is far less than that seen in the mouse. Not all proteases are equally affected under all conditions, but it is clear that protease gene expression in rodent SMG is regulated by a complex set of hormonal interactions. Receptors for androgens, estrogens, progesterone, thyroxine and glucocorticoids have been demonstrated in SMG (32), and while these hormones act predominantly at the level of mRNA transcription, they may also induce changes in translational efficiency. Additionally, hormonal re-

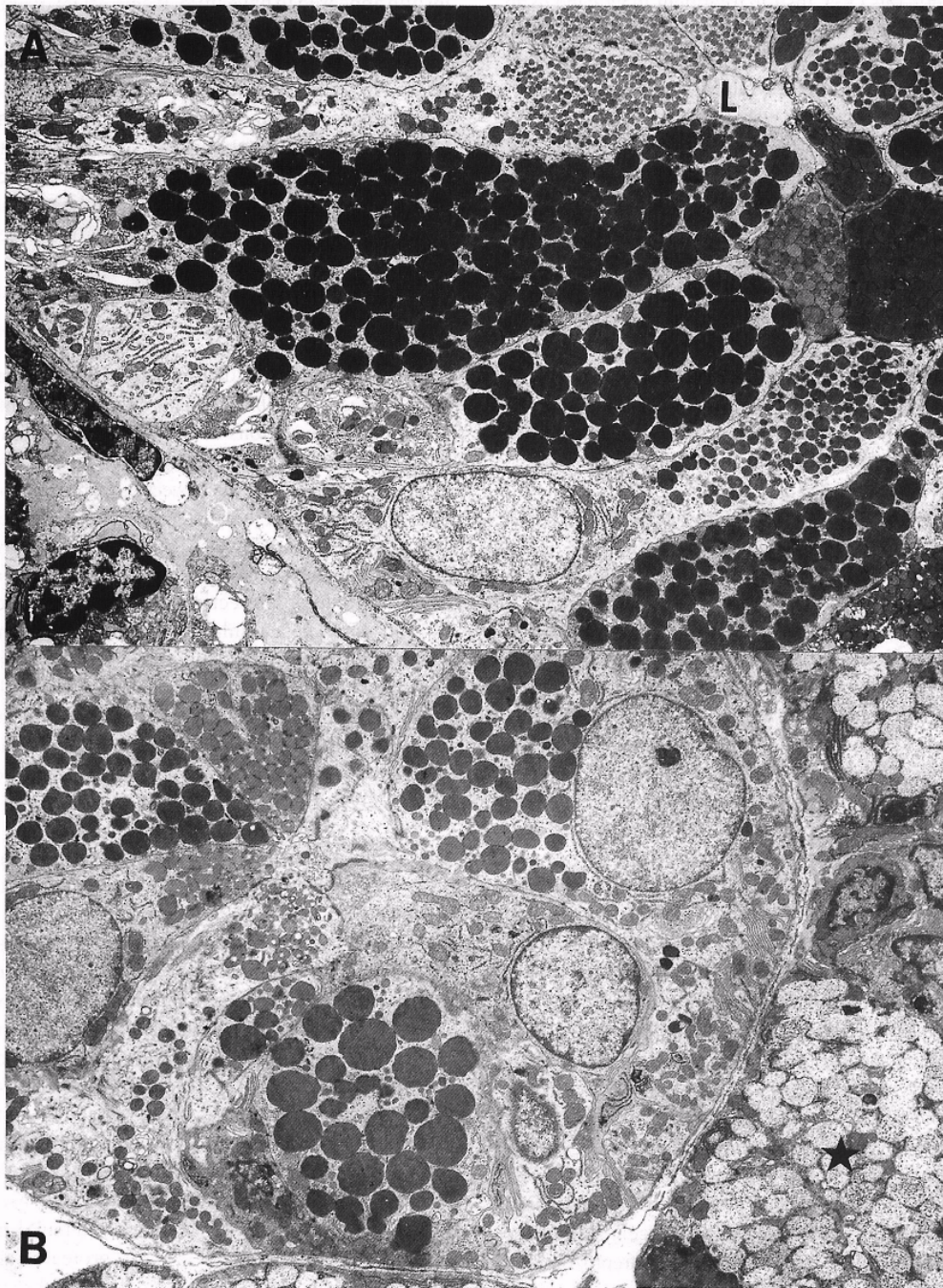


Figure 3. Electron micrograph of the submandibular gland from a control rat. This represents a portion of a granular duct, consisting of columnar cells and containing variable numbers of secretory granules of varying electron density (A). Electron micrograph of the submandibular gland from a 3-week streptozotocin-diabetic rat. Although the morphology of the duct cells appears to be relatively normal, the number of secretory granules seems to be less than in the control gland. A portion of an acinar cell (star) can also be seen (B). x 4 260 (A), x 4 460 (B). From Ref 13.

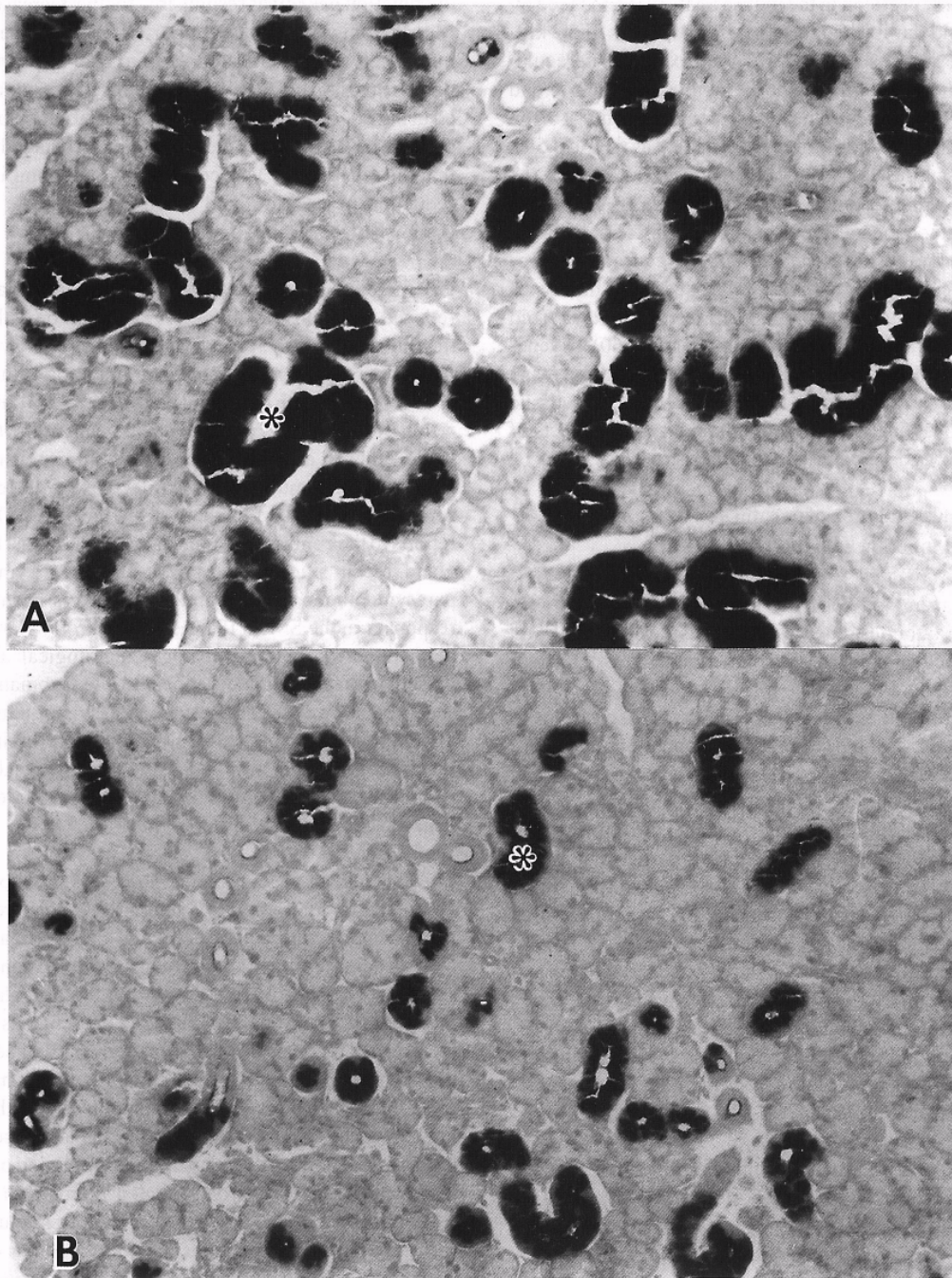


Figure 4. Light micrograph of the submandibular gland from a control rat (A). Sections stained with D-l-al-Leu-Arg-MNA and Fast Blue B to demonstrate protease activity in the secretory granules of the granular ducts (asterisks). Note the intense reaction of the substrate over the granular ducts. Light micrograph of the submandibular gland from 3-month streptozotocin-diabetic rat (B). The diabetic gland shows a considerable reduction in substrate reactivity and in the number and size of the granular ducts (asterisks) compared with the control gland, x 200 (A, B). From Ref 13.

gulation of SMG mRNA expression is quite tissue specific. For example, renin expression in the mouse SMG was enhanced by testosterone and diminished by castration, whereas in the kidney the reverse was observed (54,55). Thyroxine also upregulated the expression of renin mRNA in the mouse SMG (56,57), and the expression of kallikrein gene family members in the rat SMG was androgen and thyroid-hormone dependent as well (58,59). EGF and NGF expression in the mouse showed similar patterns of regulation by pituitary-dependent hormones (32). Each was present in greater concentrations in male than in female SMG. and castration and testosterone had opposing effects. Thyroxine and corticosteroids markedly increased the synthesis of EGF and NGF, but unlike the effects of testosterone, thyroid hormones preferentially affected NGF expression (60). In general, the levels of mRNA^{EGF} and mRNA^{NGF} parallel those of the polypeptides themselves, and hormonal control was tissue specific (61-63). Finally, estrogens antagonized the effects of testosterone on EGF and protease synthesis (64,65).

- **Neural regulation of parotid and submandibular gland function in diabetes**

Because salivary gland exocrine function is almost entirely dependent on autonomic nerves, the question has naturally arisen as to whether salivary gland function in diabetes might be affected by alterations in the neural regulation of salivary secretion. Abnormalities in acinar or granular duct sensitivity to neurotransmitters, or the development of an autonomic nerve *dysfunction per se* would be expected to have profound effects on salivary gland physiology. Neuropathy is a common complication of diabetes, encompassing a number of clinical syndromes (66), and autonomic neuropathies, in particular, result in a variety of problems affecting the digestive, respiratory, urinary and reproductive systems. Chronic hyperglycaemia, which leads to the activation of the polyol pathway, the promotion of sorbitol and fructose accumulation, Myo-inositol depletion and a slowing of nerve conduction velocity, appears to be a key factor in the development of diabetic neuropathies. However, other metabolic abnormalities, including protein glycation, superoxide radical formation, and altered amino acid and lipid metabolism, have been implicated.

In the parotid gland, sympathetic and parasympathetic nerve-stimulated salivary flow rates were reduced when compared with age-matched control animals (67). Parasympathetic impulses evoke salivary secretion largely through the activation of cholinergic receptors, and the impaired response could have reflected a decrease in the sensitivity of the acinar cells to acetylcholine. However, no differences in the threshold doses for methacholine or physalgin (a tachykinin analogue) were observed, and the volume of saliva secreted in response to each of these agonists was unaffected (68). It would appear, therefore, that the cellular mechanisms responsible for fluid secretion in the dia-

betic parotid gland are intact, and that parasympathetic nerve *dysfunction per se* was responsible for the decrease in flow rate. Morphological evidence supported this hypothesis. Electron microscopy revealed a number of axonal abnormalities in the diabetic parotid gland (67). The interpretation of results obtained using continuous sympathetic stimulation in long-term diabetic rats was more problematical, but we have hypothesized that an exaggerated vasoconstriction, resulting in a dramatic reduction in parotid gland blood flow, was the cause of the diminished sympathetic response.

The secretion of amylase from diabetic parotid glands was also reduced in response to direct nerve stimulation of the sympathetic trunk, and the reduction in amylase secretion was correlated with a decrease in the extent of degranulation of the acinar cells (69). Together with data from pharmacological and reflex secretion studies (70,71), these results suggested that diabetes leads either to a decrease in acinar cell responsiveness to α -adrenoceptor stimulation, or to a decrease in the release of neurotransmitter from sympathetic nerve terminals. Despite initial reports to the contrary (14) and in distinct contrast to the parotid gland, recent studies have shown that direct sympathetic and parasympathetic nerve stimulation evokes normal secretory responses in the SMG (19). As in the parotid gland, therefore, neither the morphological nor hematological abnormalities present in diabetic animals appear to alter submandibular fluid secretion. However, long-term diabetes (6 months) did lead to a reduction in sympathetically-stimulated protein output and secretory granule release. Thus, the effects of diabetes on protein release and fluid mobilization would appear to be independent of one another.

The decrease in SMG protein output was probably due to several factors. First, the SMG contains two different secretory compartments, the acinar cells and the granular duct cells. Neither acinar cell size nor the relative volume density of acinar secretory granules were affected, but the granular duct compartment was significantly smaller in diabetic rats. In addition, there was a reduction in the volume density of secretory granules within the granular duct cells. Finally, sympathetic stimulation caused a smaller degranulation of both the acinar and granular duct cells in diabetic rats. This last observation was particularly intriguing. Sympathetic stimulation of acinar cells leads to a β -adrenoceptor-mediated increase in cAMP, and subsequently to the exocytosis of secretory granules, whereas the secretion of polypeptides from the granular ducts is predominantly an α -adrenoceptor-mediated, Ca^{2+} -dependent event. Nonetheless, the similar effects of diabetes on these two different intracellular signaling pathways indicate that some common mechanism may be involved. One possibility is that sympathetic nerve function was impaired in diabetic animals. No morphological evidence of neuropathic changes in SMG nerves has been reported, but the levels of noradrenaline, cholineacetyl-

transferase and acetylcholinesterase are altered in both diabetic mice and rats (72-74). However, since flow rates during sympathetic stimulation were unaffected, it seems reasonable to infer that nerve impulse formation and neurotransmitter release were normal. This same conclusion pertains to the parotid gland. In addition, changes in acetylcholinesterase histochemistry and catecholamine fluorescence in *db/db* mice were found, and the differences were largely due to the effects of diabetes on the granular ducts, and a corresponding diminished neurotrophic effect of NGF (35,75). A second possibility is that the effects of diabetes on protein secretion were due to a change in adrenoceptor density or sensitivity. Thyroid hormones, for example, modulate SMG responses to a variety of neurotransmitters and neuropeptides (76-78), and circulating levels of thyroid hormones are reduced in diabetic animals. The third possibility is that the changes in membrane fatty acid profiles might alter both cAMP metabolism, as well as the generation of intracellular signaling molecules such as inositol trisphosphate and diacylglycerol, from membrane phospholipids (79). Determining which, if any, of these hypotheses is correct awaits further investigation.

SUBMANDIBULAR GLAND ENDOCRINE FUNCTIONS IN DIABETES MELLITUS

- The exocrine function of the SMG has been studied extensively, but the precise physiological roles played by the polypeptides synthesized in the granular ducts remain to be fully explored. Numerous studies have shown that the circulating serum levels of many of these biologically active substances are derived in part from the SMG, and thus the salivary glands have been hypothesized to have an endocrine function and to participate in regulating a variety of physiological processes, including reproductive tract function, neuroimmune responses and glucose homeostasis (80-83).

- **Submandibular gland as an endocrine organ**

Classically, endocrine cells and organs are characterized by their ability to sense changes in the internal environment, and as a consequence modulate the release of biologically active substances into the bloodstream. Under normal physiological conditions, endocrine cell function is regulated *via* both positive and negative feedback loops, thereby maintaining a relatively stable metabolic state. Such mechanisms have been classically termed homeostatic (83). With respect to salivary glands, responses to specific alterations in the levels of metabolites or other circulating substances remain speculative. Nonetheless, there appears to be a release of kallikreins, EGF, NGF and renin from SMG into the blood (84-86) under both resting and stimulated conditions, and experiments involving salivary gland ablation suggest that this constitutive release of biologically active polypeptides from the SMG contributes signifi-

cantly to the regulation and maintenance of homeostasis (79-83). SMG-derived EGF, for example, is thought to be necessary for normal liver regeneration after partial hepatectomy (87) and for male reproductive function (88), whereas sialoadenectomy increases the response to endotoxin (89).

- **Submandibular gland endocrine dysfunction and diabetic pathophysiology**

In contrast to homeostatic mechanisms, allostatic processes, which may be entirely inappropriate for normal function, become operative under pathological conditions (83). Under this allostatic model, changes in the exocrine or endocrine functions of salivary glands could contribute to the pathology associated with the onset of diabetes mellitus. The deficiencies in Submandibular EGF and NGF content were correlated with reductions in the plasma levels of these polypeptides, and the lower circulating levels of EGF and NGF have been hypothesized to play a role in the development of oligozoospermia (36) and diabetic neuropathy (37,38), respectively. SMG-derived NGF and EGF have also been proposed to contribute to systemic immunoregulation and neuroendocrine function, and one could speculate that diabetic complications, such as poor wound healing, may be due in part to a disruption in this neuroimmunoregulatory network (82). Alternatively, the effects of salivary constituents, which are inconsequential under normal circumstances, may be manifested in the disease state. No role for SMG-derived immunoreactive glucagon has been documented in carbohydrate homeostasis (83,90). However, salivary gland hyperglycemic factors, such as glucagon, have been suggested to exacerbate the increase in blood glucose observed in both IDDM and NIDDM animal models (91,92). Chronic hyperglycemia has been hypothesized to be a major factor in the development of vascular and neural complications in diabetes (66,93), and the inappropriate maintenance of increased serum glucose levels by extrapancreatic glucagon would therefore represent an allostatic load (83) contributing to the morbidity observed in diabetes mellitus.

CONCLUSION

- Saliva plays an important role in the protection of the oral cavity, and alterations in either salivary flow rate or composition are known to have dramatic effects on oral health. There is, however, a considerable divergence of opinion in the literature as to the extent, or even the existence, of changes in salivary composition and flow rate in insulin-treated, human diabetic patients. Nonetheless, over the past 40 years it has become clear that insulin and insulin insufficiency have both direct and indirect effects on the structure and function of the salivary glands. Insulin appears to play a direct role in the regulation of gene expression in the acinar cells, whereas its role in modulat-

ing gene expression in the granular ducts of the rodent SMG is likely to be an indirect one, mediated *via* the effects of diabetes on pituitary-dependent hormones. Diabetes may also influence salivary gland function through altered autonomic nerve function *per se*. Finally, the investigation of salivary gland structure and function in experimental diabetes has provided fundamental insights into the physiology of the salivary glands. Salivary glands, in turn, may be a valuable model system in which to study the development and pathophysiology of diabetes.

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