June 1998

Expression and Distribution of Secretory Proteins in the Rat Submandibular Gland in Experimental Diabetes

Christopher Brett Clark

Follow this and additional works at: https://opencommons.uconn.edu/sodm_masters

Recommended Citation
https://opencommons.uconn.edu/sodm_masters/27
EXPRESSION AND DISTRIBUTION OF SECRETORY PROTEINS
IN THE RAT SUBMANDIBULAR GLAND
IN EXPERIMENTAL DIABETES

Christopher Brett Clark

B.S., University of Kentucky, 1991
D.M.D., University of Kentucky, 1995

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Dental Science
at the
University of Connecticut
1998
Master of Dental Science Thesis

EXPRESSION AND DISTRIBUTION OF SECRETORY PROTEINS
IN THE RAT SUBMANDIBULAR GLAND
IN EXPERIMENTAL DIABETES

Presented by

Christopher Brett Clark, B.S., D.M.D.

Major Advisor

Arthur R. Hand, D.D.S.

Associate Advisor

Leslie S. Cutler, D.D.S., Ph.D.

Associate Advisor

Norman Tinanoff, D.D.S., M.S.

External Advisor

Maija I. Mednieks, Ph.D.

University of Connecticut

1998
ABSTRACT

Alterations in salivary composition may contribute to the increased incidence and severity of oral diseases in diabetes. Previous studies have shown changes in salivary gland structure and protein expression in experimental diabetes. The present study utilized quantitative immunocytochemistry and Western blotting to examine the effects of diabetes on secretory protein expression in the rat submandibular gland (SMG). Diabetes was induced in 2-3 month old male Fischer 344 rats by streptozotocin (STZ) injection. After 30 days, tissues were collected from one group of diabetic rats, and a second group was given insulin for 7 days. Untreated rats served as controls. Diabetic SMG acinar cells accumulated lipid and secondary lysosomes, exhibited increased secretory granule fusion and pooling of their contents, and had multiple laminae and folds of basal lamina. Quantitative immunogold labeling of secretory granules in diabetic acinar cells revealed a significant decrease (p < 0.001) in labeling density for mucin, but proline-rich proteins (PRP) and RII, a cyclic AMP receptor protein, were minimally affected. Labeling for RII in acinar nuclei was also minimally altered in diabetes. Insulin treatment had no significant effect on mucin and PRP labeling, but caused a significant decrease (p < 0.001) in RII labeling compared to control tissue. Similar changes were seen in Western blots of diabetic SMG extracts. These results indicate that STZ-induced diabetes causes variable changes in SMG secretory protein expression; insulin treatment alone fails to restore these proteins to control levels. Changes in RII levels suggest that insulin and cyclic AMP pathways may interact in regulating salivary protein expression.
Acknowledgements

Thank you to my major advisors, Dr. Art Hand and Dr. Maija Mednieks, and to my advisory committee, Dr. Leslie Cutler and Dr. Norman Tinanoff. Art and Maija’s advice, patience, and eager help throughout the past two years were invaluable. Working with them was a rewarding and unforgettable experience. Dr. Tinanoff and Dr. Cutler’s editorial assistance was very beneficial. Thank you to Christine Pearson and Mary Goss for their unlimited support and technical expertise. Thanks to Larry Hurd for his computer cleverness. I am very grateful to my parents, my brother, and Lauren Walshe for their unending and unconditional support and love.

Work was performed in the Central Electron Microscope Facility (CEMF) at the University of Connecticut Health Center. The research was supported by grant funds available to Dr. Hand (UCHC Faculty Research Grant, “Antibody to cAMP-Binding proteins of Rat Parotid Saliva”; subcontract on NIH grant DE09428 to L. Mirels, University of California, Berkeley, “Regulation of Submandibular Gland Gene Expression”).
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approval Page</td>
<td>ii</td>
</tr>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Objectives</td>
<td>6</td>
</tr>
<tr>
<td>General Goal</td>
<td>6</td>
</tr>
<tr>
<td>Specific Objectives</td>
<td>6</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>6</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>8</td>
</tr>
<tr>
<td>Animals and Animal Care</td>
<td>8</td>
</tr>
<tr>
<td>Induction and Assessment of Diabetes</td>
<td>8</td>
</tr>
<tr>
<td>Serum Glucose Measurements</td>
<td>9</td>
</tr>
<tr>
<td>Tissue Preparation for Electron Microscopy</td>
<td>9</td>
</tr>
<tr>
<td>Morphological Analysis</td>
<td>10</td>
</tr>
<tr>
<td>Antibodies</td>
<td>10</td>
</tr>
<tr>
<td>Immunogold Labeling</td>
<td>10</td>
</tr>
<tr>
<td>Quantitation of Immunogold Labeling</td>
<td>11</td>
</tr>
<tr>
<td>Tissue Preparation for Gel Electrophoresis</td>
<td>11</td>
</tr>
<tr>
<td>SDS-Polyacrylamide Gel Electrophoresis and Western Blotting</td>
<td>12</td>
</tr>
<tr>
<td>Results</td>
<td>14</td>
</tr>
<tr>
<td>Serum Glucose and Body Weight</td>
<td>14</td>
</tr>
<tr>
<td>Morphology</td>
<td>14</td>
</tr>
<tr>
<td>Quantitative Immunocytochemistry</td>
<td>15</td>
</tr>
<tr>
<td>SDS-Polyacrylamide Gel Electrophoresis and Western Blotting</td>
<td>15</td>
</tr>
<tr>
<td>Discussion</td>
<td>17</td>
</tr>
<tr>
<td>Tables</td>
<td>26</td>
</tr>
</tbody>
</table>
List of Tables

Table 1. The effects of experimental diabetes and insulin treatment on
serum glucose levels and body weight ........................................... 27

Table 2. Morphometric analysis of immunogold labeling of submandibular
gland acinar secretory granules for mucin and PRP ....................... 28

Table 3. Morphometric analysis of immunogold labeling of submandibular
gland acinar secretory granules and nuclei for RII ....................... 29

Table 4. The effects of experimental diabetes and insulin treatment on
submandibular gland protein expression measured from
Western blots ............................................................................. 30

Table 5. The effects of experimental diabetes and insulin treatment on
parotid gland protein expression measured from
Western blots ............................................................................. 31
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Electron micrographs of control rat submandibular gland</td>
<td>38</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Electron micrographs of diabetic rat submandibular gland</td>
<td>39-40</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Electron micrographs of insulin-treated diabetic rat submandibular gland</td>
<td>41</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Immunogold labeling of acinar secretory granules with anti-SMG mucin antibody</td>
<td>42</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Immunogold labeling of acinar secretory granules with anti-PRP antibody</td>
<td>43</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Immunogold labeling of acinar secretory granules with anti-RII antibody</td>
<td>44</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Immunogold labeling of acinar nuclei with anti-RII antibody</td>
<td>45</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Western blot of anti-SMG mucin</td>
<td>46</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Western blot of anti-PRP</td>
<td>46</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Western blot of anti-RII</td>
<td>46</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Densitometric scans of anti-SMG mucin in submandibular gland extracts</td>
<td>47</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Densitometric scans of anti-PRP in submandibular gland extracts</td>
<td>48</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Densitometric scans of anti-RII in submandibular gland extracts</td>
<td>49</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Densitometric scans of anti-PRP in parotid gland extracts</td>
<td>50</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Densitometric scans of anti-RII in parotid gland extracts</td>
<td>51</td>
</tr>
</tbody>
</table>
INTRODUCTION

Diabetes mellitus is a complex, multifactorial, genetically derived endocrine disorder. It refers to an abnormality in glucose metabolism due to insulin deficiency, impaired utilization of insulin, or an error in insulin metabolism. An elevated blood glucose level (hyperglycemia) is the common characteristic. The disorder is evident in two forms: type I (insulin-dependent diabetes mellitus [IDDM]) or type II (non-insulin-dependent diabetes mellitus [NIDDM]). NIDDM, representative of impaired insulin function and the more common form, results from defects in the insulin molecule or from altered cellular insulin receptors. IDDM, the more severe form, is caused by the destruction of the insulin-producing beta cells of the pancreatic islets of Langerhans. The pathophysiology may involve an autoimmune or virally mediated destructive process.

Insulin, an anabolic hormone, promotes glucose uptake by inducing the rapid translocation of specific glucose carrier proteins from an intracellular pool to the plasma membrane (Espinal, 1987; Smith, 1987). Insulin plays a major role in regulating several metabolic pathways, including fatty acid synthesis, carbohydrate metabolism and glycogen storage, amino acid transport, and the synthesis of DNA, RNA, and protein (Smith, 1987).

The classic signs and symptoms of diabetes mellitus are more common in IDDM. They include the triad of increased thirst (polydipsia), increased hunger (polyphagia), and frequent urination (polyuria) together with weight loss, weakness, and fatigue. Systemic ketosis or acidosis leading to diabetic coma or death can result from the absence of insulin supplementation. Even with insulin supplementation some diabetic patients can develop chronic systemic complications like retinopathy, nephropathy, sensory neuropathy, and cardiovascular and cerebrovascular atherosclerotic diseases (Nathan, 1993).

Diabetes mellitus has also been shown to cause oral complications. Caries and periodontal disease appear to occur with a higher frequency in untreated or poorly controlled diabetic patients (Cutler and Valachovic, 1982; Loe and Genco, 1995).
Enlargement of the parotid gland (Russotto, 1981) has been noted, but it too seems to be more common in poorly controlled diabetic patients (Ben-Aryeh et al., 1988, 1993; Marder et al., 1975). Changes in salivary flow and composition (Ben-Aryeh et al., 1988, 1993; Beeley, 1991; reviewed in Rossie, 1993) have been demonstrated. Lower resting salivary flow rates were found in IDDM patients as compared to the NIDDM and control groups, and the total protein concentration in whole saliva of diabetic patients was higher (Ben-Aryeh et al., 1988, 1993). Xerostomia, or dry mouth, is another common complaint of poorly controlled diabetics.

Insulin-dependent diabetes mellitus can be induced in rats by the administration of streptozotocin (STZ), a broad-spectrum antibiotic extracted from Streptomyces acromogenes. Streptozotocin has a specific cytotoxic effect on pancreatic insulin-producing B-cells. Degranulation occurs as early as 1 hour after STZ administration and necrosis of the B-cells is seen after 7 hours (Junod et al., 1967). Furthermore, proinsulin biosynthesis and glucose-stimulated insulin secretion are immediately and permanently suppressed after a brief exposure to the drug (Wilson et al., 1984). Pancreatic glucagon-producing cells, however, are not affected. Rakieten et al. (1963) were the first to report that STZ causes diabetes in rats and dogs. During the past 35 years many studies have confirmed this finding and found that rats treated with STZ display many of the features seen in human subjects with uncontrolled diabetes, including hyperglycemia, polydipsia, polyuria, and weight loss (Ar’Rajab and Ahren, 1993; Tomlinson et al., 1992).

The effects of experimental diabetes on major salivary gland morphology have been demonstrated. In young rats, diabetes markedly affects the growth and development of the parotid and submandibular glands. In adult rats there is a marked increase in parotid gland weight (Anderson, 1983) while there is a significant decrease in the submandibular gland weight (Anderson et al., 1993, 1994). In both the rat parotid and submandibular glands, diabetes causes rapid accumulation of lipid within the acinar cells (Anderson, 1983; Hand and Weiss, 1984; Anderson and Garrett, 1986; Reutervig et al., 1986). The amount of
lipid in the submandibular gland, however, appears to be less and the droplets tend to be smaller and located only in the seromucous acini and intercalated duct cells (Anderson and Garrett, 1986; Anderson et al., 1994). In the diabetic parotid gland, acinar cells contain crystalloid lysosomes (Hand and Weiss, 1984) and duct cells contain large vacuoles resulting from the endocytosis of acinar secretory proteins present in the saliva (Lotti and Hand, 1988). In the diabetic submandibular gland, Cutler et al. (1979) and Takai et al. (1983) found pooling of secretory material and an increase in the number of autophagic vacuoles and secondary lysosomal structures in acinar cells, and extensive cellular degeneration and replacement of the secretory cells by fibrous connective tissue. Anderson et al. (1993, 1994) found similar changes in some of the acinar and granular duct cells; however, most of the cells appeared relatively normal and the glands showed no evidence of fibrous degeneration or irreversible cellular damage. They noted the greatest effect of STZ-induced diabetes was the significant reduction in the volume density of the granular duct cells and the secretory granules they contain. The volume density of the acini increased while that of the secretory granules was unaffected.

In previous studies the effects of experimental diabetes, insulin, parasympathetic and sympathetic stimulation on the rate of protein synthesis and salivary secretion in the rat submandibular gland were examined. Peroxidase, a secretory enzyme, and the incorporation of $^3$H-leucine into trichloroacetic acid (TCA)-precipitable proteins were decreased in alloxan diabetes and restored to control levels after insulin supplementation (Anderson and Shapiro, 1980). This implied that insulin had a direct effect on the rate of protein synthesis. Furthermore, it appeared that the regulation of protein synthesis involved a bi-directional system in which alpha-adrenergic and cholinergic stimulation inhibited amino acid incorporation, whereas beta-adrenergic agonists and insulin enhanced the rate of synthesis (Baum et al. 1984; Anderson, 1988). Matsuura and Hand (1991), however, found that chronic beta-adrenergic stimulation had selective effects on the expression of several submandibular gland proteins. Namely mucin, proline-rich proteins
(PRP), and type 2 cystatin increased, glutamine/glutamic acid-rich proteins (GRP) decreased, and protein SMG-C was minimally affected.

Anderson et al. (1993) also studied the effects of diabetes on sympathetic versus parasympathetic nerve stimulation on the secretory response in the rat submandibular gland. In 3 and 6 month old diabetic rats, the sympathetic flow rate was greater than controls, but the protein concentration was reduced. The decrease in salivary protein concentration was accompanied by a reduction in secretory granule release from acinar and granular duct cells. No consistent differences in flow rate, protein concentration, protein output, or secretory granule release, however, were noted following parasympathetic stimulation in diabetic rats versus controls. Anderson et al. concluded that only the type of nerve stimulation rather than diabetes had an effect on salivary flow rate or protein release in the submandibular gland. Additionally, Anderson et al. (1995) found that the secretion of proteins by the acinar cells and granular duct cells of the submandibular gland varies according to the degree and type of neural stimulation - sympathetic versus parasympathetic. From these studies, it seems that sympathetic (beta-adrenergic) responses are more affected in diabetes than parasympathetic responses.

Several studies of experimental diabetes have demonstrated changes in salivary protein composition in the major salivary glands. In the parotid gland, amylase (Anderson and Johnson, 1981; Anderson, 1983, 1987; Kim et al., 1990; Zebrowski and Brimmer, 1978) and amylase mRNA levels (Kim et al., 1990) decreased in diabetic rats and were restored with insulin treatment. Other secretory proteins, such as parotid secretory protein (PSP) (Lotti and Hand, 1988; Szczeaphaelski et al., 1998) and PRP (Anderson and Johnson, 1981; Anderson, 1991; Szczepanski et al., 1998), showed a significant decrease in diabetic rats while insulin reconstitution had little or no effect. In the submandibular gland, Chan et al. (1993) showed that all activities of the proteinases, tissue kallikrein and tonin, were significantly reduced in diabetic rats. Insulin treatment restored tissue kallikrein activity, whereas the activity of tonin was unchanged.
The effects of experimental diabetes on the expression and distribution of the secretory proteins in the rat submandibular gland have not been studied. The proteins that will be examined can be divided into two groups: adult acinar cell secretory and cellular regulatory proteins. The majority of proteins produced in the rat submandibular gland originate from the adult acinar cells. Therefore, proteins such as SMG mucin and PRP will be evaluated. A cyclic AMP receptor protein (cARP) will be studied to determine if cyclic AMP-mediated reactions are involved in regulating salivary protein expression in diabetes. Specifically, the type II regulatory subunits (RII) of cyclic AMP-dependent protein kinase (protein kinase A [PKA] ) will be examined. The cARP are critical components of the major intracellular pathway regulating secretion and gene expression in salivary glands (Flockhart and Corbin, 1982; Lohmann and Walter, 1984; Krebs, 1992). These proteins have been found in secretory granules (Mednieks et al., 1987) and are secreted in rat saliva (Mednieks and Hand, 1984) and human saliva (Mednieks et al., 1993). Type II R subunits have specific cellular functions as protein and DNA binding proteins. Additionally, RII has been shown to bind to actin and may be involved in the cytoskeletal activity of secretory cells (Scott et al., 1990). The specific function of cARP in saliva, however, is not presently known.

Although some proteins of rat submandibular saliva like GRP have no direct counterpart in human saliva, others, such as mucin glycoprotein, PRP, and RII share many similarities with their human analogs. These proteins have many functions that probably are important for oral health in both species. If diabetes alters the synthesis and expression of these proteins, an individual’s oral health could be compromised.

The fundamental hypothesis, therefore, is that the synthesis and cellular distribution of secretory proteins are altered as a consequence of diabetes, and are measurable as changes in the expression of specific proteins in the submandibular gland.
OBJECTIVES

1. General Goal:

   To determine the effects of experimental diabetes on the expression and cellular distribution of secretory proteins in the submandibular gland of control, diabetic, and insulin-treated diabetic rats.

2. Specific Objectives:

   A.) Compare the morphology of the rat submandibular gland in control, diabetic, and insulin-treated diabetic groups.

   B.) Compare the expression and distribution of secretory proteins (mucin, PRP, and RII) in the rat submandibular gland of control, diabetic, and insulin-treated diabetic groups using quantitative electron microscopic immunocytochemistry.

   C.) Identify and characterize the expression of these secretory proteins in tissue extracts of the rat submandibular gland of control, diabetic, and insulin-treated diabetic groups using SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

3. Experimental Design

   A.) The rat has served as an animal model in numerous studies. The experimental system employed in this study was salivary gland tissues of streptozotocin-treated rats. Many features of this system are similar to those seen in human subjects with uncontrolled diabetes mellitus (Ar’Rajab and Ahren, 1993; Tomlinson, 1992). The morphology of the salivary glands and the location and functions of their proteins are well documented.

   B.) Both quantitative electron microscopic immunocytochemistry and Western blotting were used for comparison of the expression of secretory proteins. In immunocytochemistry the detection of a whole protein versus its proteolytic fragments cannot be easily distinguished, while in PAGE and Western blotting it can. Therefore, this
approach can help determine whether changes seen in the expression of secretory proteins are due to effects on synthesis or degradation.
MATERIALS AND METHODS

Animals and Animal Care

Two to three-month old, viral antibody-free, male NIA-Fischer 344 rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). The housing, care, and use of the animals were in accordance with NIH guidelines, and all procedures involving animals were reviewed and approved by the University of Connecticut Health Center Animal Care Committee. The rats were housed in plastic microisolator cages and randomly assigned to control, diabetic, and insulin reconstitution groups. They were fed autoclaved rat chow and water ad libitum and allowed to acclimate for 3-5 days prior to inducing diabetes. The experimental animal work involving the induction of diabetes, the subsequent administration of insulin, and the tissue collection and preparation was previously performed by Szczepanski et al. (1998). Tissues from the control animals were collected and processed specifically for this study.

Induction and Assessment of Diabetes

Diabetes was induced by intraperitoneal injection of 35-40 mg/kg streptozotocin (STZ, Sigma Chem. Co., St. Louis, MO) in 0.01 M citrate buffer, pH 4.5. The rats were observed daily, and the development and course of the diabetes were followed by weighing the rats weekly and monitoring blood glucose with the One-Touch II glucose meter (Lifescan, Milpitas, CA), using a drop of blood from the lateral tail vein. Diabetic animals failed to gain, or lost weight, exhibited polyuria and polydipsia within a few days, and had blood glucose levels routinely exceeding 400 mg/dL. Beginning at one month after the induction of diabetes, one group of animals was treated with Lente insulin (10 Units/day, s.c.) daily for 7 days. Blood glucose levels of insulin-treated animals were monitored periodically to evaluate the insulin replacement therapy.
**Serum Glucose Measurements**

Blood samples were drawn from the hearts of anesthetized rats, prior to killing. Serum was separated from clotted blood samples and stored at -70 °C. Serum glucose levels were determined by the hexokinase method, using an assay kit and procedures from Sigma Chemical Co. (St. Louis, MO).

**Tissue Preparation for Electron Microscopy**

Submandibular glands were fixed by vascular perfusion of anesthetized rats (sodium pentobarbital, 50 mg/kg body weight, i.p.) with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, then excised and immersed in fresh fixative solution. After fixation for 1 hour, the glands were rinsed and stored briefly at 4 °C in 0.1 M cacodylate buffer, pH 7.4. Small submandibular gland tissue pieces about 1 mm³ were randomly selected, dehydrated in ethanol, and substituted with propylene oxide. Multiple sectioning blocks were prepared by embedding in PolyBed (Polysciences, Warrington, PA) and polymerizing at 60 °C. For morphological study, some tissue was postfixed in 1% osmium tetroxide and stained with 0.5% aqueous uranyl acetate prior to embedding in PolyBed.

Several PolyBed blocks were randomly selected. One µm thick sections were cut with glass knives, stained with methylene blue-Azure II, and examined in the light microscope for orientation. Tissue blocks containing the selected areas were sectioned to a thickness of 70-90 nm using a diamond knife, and the sections were collected on 300 mesh copper grids for morphological study. Another series of sections was collected on uncoated nickel grids for immunogold labeling.
Morphological Analysis

Acinar cells of control, diabetic, and diabetic insulin-treated animals were examined using a JEOL 100 CX transmission electron microscope (TEM) at low magnification (1,600 to 2,600X) for morphological features. Morphological characteristics and changes were then examined at higher magnifications, photographed, and printed at final magnifications ranging from 4,700X to 47,000X.

Antibodies

For the adult acinar cell proteins, anti-SMG mucin (mouse monoclonal antibody) was obtained from Dr. L. Tabak, University of Rochester (Moreira et al., 1989), and anti-PRP (rabbit antibody) was a gift from Dr. D. Carlson, University of California, Davis (Ziemer et al., 1982). An antibody to the cellular regulatory protein, RII (rabbit antibody), was prepared by Dr. M.I. Mednieks, University of Illinois-Chicago/University of Connecticut Health Center.

Immunogold Labeling

Immunogold labeling was performed as described by Hand (1995). For mucin and PRP, one grid from each animal was randomly selected for each antibody and treated with 1% BSA (bovine serum albumin)-1% instant milk in phosphate-buffered saline (PBS) for 30 minutes at room temperature to block non-specific binding of antibodies. The grids were transferred to primary antibody diluted in 1% BSA with 5% normal goat serum (NGS) in PBS for overnight incubation at 4°C. For RII, one grid from each animal was randomly selected and treated with 2X Teloblot (4% w/v non-mammalian proteins, 1 µM sodium azide, 0.01% Tween-20 detergent) for 30 minutes at room temperature, and transferred to primary antibody diluted in 2X Teloblot for overnight incubation at 4°C. The bound immunoglobulins were visualized by incubation for 60 minutes at room temperature.
with 10 nm gold labeled goat anti-rabbit IgG or goat anti-mouse IgG (Amersham, Arlington Heights, IL). After rinsing with PBS and distilled water, the sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100 CX TEM. Immunocytochemical controls included substitution of preimmune or non-immune sera or 1% BSA-5% NGS for the primary antibody.

**Quantitation of Immunogold Labeling**

Cells were randomly selected on the microscope screen at low magnification (5,000X) so that gold particles were not visible, then photographed at approximately 16,000X, and printed at a final magnification of 43,000X. For mucin and PRP in each animal, ten fields containing secretory granules and three fields containing nuclei were photographed. For RII, ten fields of each organelle were photographed. Quantitative analyses of the immunogold labeling of intracellular compartments were done on the prints using a digitizing tablet and SigmaScan software (Jandel, Corte Madera, CA). Non-specific binding was evaluated over areas of plastic (2 per grid) adjacent to tissue. Analysis of variance (ANOVA), using the Statistical Program for Social Scientists (SPSS) software, was applied to determine if differences among mean labeling densities of control, diabetic, and insulin-reconstituted samples were statistically significant. Specific differences between the sample means were tested for statistical significance using Bonferroni and Tukey post-hoc tests.

**Tissue Preparation for Gel Electrophoresis and Western Blotting**

The rats were euthanized with CO₂ and killed by cardiac transsection. Glands were then dissected, trimmed of extraneous tissue, cut in ~ 0.5 cm³ pieces, frozen in liquid nitrogen, and stored at -70 °C. Prior to assay, the randomly selected tissue samples were thawed in distilled water and an equal volume of 0.1 M Tris buffer, pH 6.8, containing 0.15 M KCl and 1 µM each of phenylmethylsulfonyl fluoride and benzamidine. The
tissues were homogenized with a Polytron probe, transferred to micro-centrifuge tubes, and centrifuged at 10 kG. Supernatants containing soluble cell components were recovered and diluted to appropriate protein concentrations (approximately 0.5 mg/ml total protein) in sample buffer (0.1% bromphenol blue, 10% SDS, distilled water, glycerol, mercaptoethanol), heated to 90 °C, and stored at -70 °C.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting**

The samples for gel electrophoresis were placed in a boiling water bath for 5 minutes and cooled to room temperature. They were applied to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels to separate the proteins according to molecular size (Laemmli, 1970). A Rainbow marker™ (Amersham RPN 756, Arlington Heights, IL) with molecular weights ranging from 14.3 to 220 kD was used as the standard. The gels were run at 50 volts for 2.5 to 3 hours in the presence of 10⁻⁸ M ethylenediaminetetraacetic acid (EDTA) for calcium chelation to prevent streaking of the submandibular gland extract that probably occurred as a result of mucin complexes. Banding patterns of parotid gland extract were more distinct and had minimal streaking even if they were run at higher voltages and for shorter times. The proteins were transferred to nitrocellulose membranes by electroblotting (Towbin et al., 1979). The nitrocellulose membrane was stained with 0.5% Ponceau S dye in 5.0% trichloroacetic acid to ensure that efficient protein transfer had occurred and to help determine total protein loaded for each lane. For immunoblotting, the membrane was washed with PBS and distilled water for 30 minutes to remove the Ponceau S. The membrane was incubated in 5X Teloblot or 1% BSA-1% instant milk for 1 hour to block non-specific binding sites.

Detection of the proteins on the membrane was achieved by an indirect method. The membrane was incubated in a solution of unlabeled primary antibody and PBS-(0.5X)Teloblot or PBS-1% BSA at room temperature for 1 hour and then at 4 °C
overnight. The primary antibodies (anti-mucin, anti-PRP, anti-RII) used for immunogold labeling were also employed for Western blotting. The membrane was washed with 3 changes of PBS to remove unbound primary antibody. A secondary antibody labeled with horseradish peroxidase in a dilution of PBS-(0.5X)Teloblot or PBS-1% BSA was added, and the membrane was incubated for 1 hour at room temperature. The secondary antibodies utilized were goat anti-mouse polyvalent immunoglobulins and goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO). The membrane was washed with 3 changes of PBS. Diaminobenzidine (DAB) 6 mg, 10 ml 50 mM Tris, pH 7.6, and 15 µl 30 % hydrogen peroxide were added to the membrane and incubated at room temperature for 1-5 minutes. A brown reaction product identified the specific protein band on the membrane.

A Ponceau S record of total protein loaded for each lane and immunoblots of each protein (mucin, PRP, RII) were scanned using OFOTO software, and band density measurements were done using NIH Image software. The integrated density, area under the curve, and background measurements in the densitometric scans were tabulated for quantitation of the total protein and each specific protein (not shown). The expression of the specific proteins was calculated from these tables and is shown as ratio of each specific protein measured by Western blotting to the total protein applied to the PAGE lane (Tables 4,5).
RESULTS

Serum Glucose and Body Weight

Polydipsia, polyphagia, and polyuria were seen in all diabetic rats. Mean serum glucose levels in animals one month after the induction of diabetes increased over 3.5 fold compared to control animals (Table 1), while mean body weights of diabetic rats decreased 21.5% from initial weights. After insulin treatment for 7 days, mean serum glucose levels were elevated only 2.5 fold compared to control animals. Insulin treatment also resulted in a mean increase of 7.40% from the animals’ diabetic weight.

Morphology

The morphological features of the submandibular glands of control animals were similar to those described in previous studies (Anderson et al., 1993,1994; Cutler et al., 1979; Takai et al.,1983). The seromucous acinar cells contained electron lucent secretory granules of varying size with a finely filamentous content in the apical two-thirds of their cytoplasm (Fig. 1a,b). Some secretory granules were occasionally fused with one another. Nuclei, endoplasmic reticulum, and Golgi apparatus were positioned in the basal one-third of the cell.

One month after the induction of diabetes, the acinar cells displayed several abnormalities. Lipid accumulation in the basal cytoplasm was the most common observation, while folding of the basal lamina and multiple laminae were also prominent (Fig. 2a, b). In addition, the presence of secondary lysosomes and the coalescence of secretory granules and pooling of their contents were noted (Fig. 2c,d). The acinar cells of insulin-treated diabetic animals showed no lipid accumulation and had relatively normal morphological features (Fig. 3a,b).
Quantitative Immunocytochemistry

Immunogold labeling of rat submandibular gland acinar secretory granules with anti-mucin antibody was significantly reduced (p < 0.001) in diabetes. No effect on the labeling density was seen with insulin treatment (Fig. 4a,b,c; Table 2). Labeling of secretory granules with antibody to PRP was reduced in the diabetic and insulin-treated diabetic tissues, but the differences were not statistically significant (Fig. 5a,b,c; Table 2). Immunogold labeling of acinar secretory granules and nuclei with anti-RII antibody was unaffected in diabetes. A significant decrease (p < 0.001) in the labeling density of granules and nuclei occurred in the insulin-treated diabetic tissue (Fig. 6a,b,c; Fig. 7a,b,c; Table 3). Background labeling density was low (0.25-0.86 gold particles/μm²) and did not differ among the three groups for mucin and PRP antibodies. The background labeling density for RII antibody in the insulin-treated diabetic tissue (8.46 gold particles/μm²) was significantly increased (p < 0.01) compared to the control tissue (4.66 gold particles/μm²).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Submandibular gland tissue extracts were analyzed using Western blots of proteins separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Antibodies against SMG-mucin, PRP, and RII recognized bands of approximately 114, 25-35, and 50 kD, respectively (Fig. 8,9,10). Densitometric analysis of Western blots showed a decrease in the integrated densities of mucin, PRP, and RII for the diabetic rats and a virtual return to control levels for the insulin-treated diabetic groups (Fig. 11,12,13).

The integrated density, area under the curve, and corrections for background in scans of each specific protein immunoblot and the total protein record were used to quantitate the expression of each protein. Diabetic rats had a significant decrease in the expression of mucin, while PRP and RII were minimally reduced (Table 4). Insulin treatment partially restored the concentration of mucin to control levels, returned PRP fully to control levels, and increased RII above control levels.
Electrophoretic separation and Western blotting were also used to analyze parotid gland tissue extracts (Fig. 8,9,10). The expression of PRP and RII were decreased in the diabetic rats (Table 5). In the insulin-treated diabetic group, PRP expression was almost restored to control levels, while RII levels displayed a further decrease. As expected, mucin was not found in parotid extracts. Densitometric scans showing integrated densities for PRP and RII in control, diabetic, and insulin-treated diabetic groups (Fig. 14,15) exhibited changes similar to their calculations for protein expression (Table 5).
DISCUSSION

The classic triad signs of insulin-dependent diabetes mellitus (polydipsia, polyphagia, and polyuria) were seen in all diabetic rats. Similar to the results reported in other studies (Anderson and Shapiro, 1979; Anderson et al., 1993, 1994; High et al., 1985), a reduction in body weight and an increase in mean serum glucose levels in diabetic animals were also noted. After insulin treatment for 7 days, body weight increased and mean serum glucose levels decreased compared to the animals’ diabetic measurements. The rat model of IDDM in our study, therefore, has the major manifestations seen in human diabetes.

The submandibular gland of diabetic rats exhibited morphological changes similar to those described in previous studies. Lipid droplets in the basal cytoplasm were evident in many seromucous acinar cells. Additionally, pooling of secretory material and the presence of secondary lysosomes were observed, but autophagic vacuoles or extensive cellular degeneration as noted by Cutler et al. (1979) and Takai et al. (1983) were not seen in the electron microscopic fields examined. These differences may be due to varying experimental conditions between our study and theirs that include the strains of rats (Fischer vs. Sprague-Dawley) and the time at which the diabetic tissues were observed (30 days vs. 4 hrs. to 21 days). Also, examining larger areas with light microscopy could have revealed the presence of necrotic secretory cells and connective tissue replacement. Our observations, however, were similar to those of Anderson et al. (1993, 1994) except we noted that most of the acinar cells present in the diabetic gland showed at least a few characteristic morphological changes.

In addition, extra basal lamina material deposited around the acinar cells was observed in our study. Previous studies of the diabetic rat submandibular gland have not reported the presence of excessive basal lamina material. These findings resembled changes seen by Hand and Weiss (1984) and Szczepanski et al. (1998) in the diabetic rat parotid gland. Basal lamina alterations have also been found in human diabetic studies.
involving the kidney (Huang, 1980) and muscle (Kilo et al., 1972). At present it is not known whether the salivary gland changes in human diabetes include basement membrane alterations. A study by Rohrbach et al. (1982) found that the synthesis and quantity of basement membrane components such as Type IV collagen and laminin are increased in diabetes, while the synthesis and sulfation of basement membrane proteoglycans are reduced. Studies by Kanwar and Farquhar (1979) and Kanwar et al. (1980) suggest that the highly anionic proteoglycans account for the permeability and filtration properties of the basement membrane. It has been hypothesized that cells compensate for the defective nature of the basement membrane in diabetes by enhancing synthesis that results in the increased thickness and layering (Rohrbach et al., 1982). As in the kidney, the salivary gland epithelium constitutes a major site of fluid and electrolyte transfer (Schneyer et al., 1972). Therefore, the basement membrane alterations seen in STZ- induced diabetes could cause changes in salivary flow and composition which could influence the function of the submandibular gland.

The morphology of the diabetic rat submandibular gland after insulin treatment has rarely been documented. In our study we observed that the seromucous acinar cells, after only 7 days of insulin treatment, had no lipid accumulation or other morphological features that were characteristic in the diabetic rat. This supports the observations of Morris et al. (1992) who found that the lipid content and the fatty acid profiles of all major salivary glands return to normal after 7 days of insulin treatment.

Quantitative immunocytochemistry has been used to assess the effects of various experimental conditions on the expression and distribution of secretory proteins in several tissues, including the parotid gland (Lotti and Hand, 1988; Szczepanski et al., 1998) and pancreas (Grégoire and Bendayan, 1986,1987) in experimental diabetes. This is the first study, however, to apply this methodology to the rat submandibular gland in experimental diabetes. Previous studies have used submandibular gland homogenates and chemical assays to analyze the activities of secretory enzymes. Anderson and Shapiro (1980) found
that peroxidase activity was reduced in alloxan diabetes and restored to control levels after insulin treatment. Chan et al. (1993) noted that all activities of the proteinases, tissue kallikrein and tonin, were significantly decreased in STZ-diabetic rats, while insulin treatment restored tissue kallikrein activity and had no effect on tonin activity.

Immunogold labeling of submandibular gland sections with antibodies to individual secretory proteins revealed specific responses of the seromucous acinar cells to diabetes. Mucin was the only secretory protein to have a significant decrease (p < 0.001) in granule labeling density. RII granule labeling density was not affected in diabetes, while an insignificant decrease was seen in PRP labeling. Our study has revealed that the expression of individual submandibular secretory proteins are variably affected in diabetes. Szczepanski et al. (1998) observed similar variability in the effect of diabetes on the expression of secretory proteins in the parotid gland. They found that the granule labeling density of amylase, PRP, and parotid secretory protein (PSP) are significantly decreased, while the labeling density of RII and acidic epididymal glycoprotein (AEG) were minimally affected.

The presence of mature and active proteins in a cell is the sum of the complex steps in gene expression: 1) synthesis of the primary transcript; 2) turnover and processing of the primary transcript; 3) transport of the mRNA out of the nucleus and turnover in the cytoplasm; 4) translation; and 5) modification and protein turnover. Therefore, factors at any of these steps may control the abundance of the mRNAs and play an important role in the regulation of protein levels (Kousvelari and Tabak, 1991).

The regulation of protein levels by insulin involves both the transcription and translation processes. First, insulin must bind to the extracellular domain of an insulin receptor within the plasma membrane of a target cell. The receptor activates an intracellular tyrosine protein kinase that phosphorylates a number of intracellular proteins (substrates). Insulin receptor substrate (IRS)-1 activates phosphatidylinositol 3-kinase which in turn
activates several signaling pathways which stimulate the transcription of certain genes and regulates the translational process of protein synthesis (Moule and Denton, 1997).

In our study, insulin treatment had no significant effect on the restoration of mucin, PRP, or RII granule labeling densities to their control levels. In fact, a significant decrease (p < 0.001) was seen in the granule labeling density of RII for insulin-treated diabetic animals compared to control animals. Variability is observed in how insulin treatment affects the expression of each specific secretory protein. Chan et al. (1993) and Szczepanski et al. (1998) made similar observations in their studies. Szczepanski et al. (1998) found that in the parotid gland insulin treatment had no effect on PRP, PSP, or AEG levels, while a significant decrease occurred in RII granule labeling.

One reason why insulin alone did not restore the secretory proteins to their control levels is that other hormones or biological factors could be involved in the expression of these proteins. Many genes in other tissues such as pancreatic amylase, pituitary growth hormone, and liver pyruvate kinase are regulated by insulin in conjunction with cyclic AMP, glucocorticoids, thyroid hormones, and/or androgens. The effect of cyclic AMP on these genes is often opposite of the insulin effect. Under normal physiological conditions, these regulators coexist and interact to influence transcription (Meisler and Howard, 1989).

Considerable evidence suggests that in rodents the biosynthesis, processing, and secretion of salivary secretory proteins are also mediated by the sympathetic nervous system. β-adrenoreceptor stimulation has a profound effect on the regulation of mRNA levels of many salivary gland genes like amylase, PRP, PSP, and tissue kallikrein. The mRNA for most of these genes are regulated by cyclic AMP (Kousvelari and Tabak, 1991). Typical examples of genes for which transcription is regulated by cyclic AMP or agents that increase cyclic AMP include the liver enzyme phosphoenolpyruvate carboxykinase (Sasaki et al., 1984) and the hypothalamic peptide somatostatin (Gonzalez and Montminy, 1989). It has been found that cyclic AMP mediates the hormonal induction
of these genes by a conserved cyclic AMP response element (CRE) present in their promoter region (Montminy, 1987).

Another reason for the failure of insulin to restore the secretory proteins to control levels could be due to the protocol used for the administration of insulin to the diabetic rats. They were given single daily insulin injections which probably resulted in widely varying serum insulin levels throughout the day. Since mean serum glucose levels of our insulin-treated diabetic rats didn’t return fully to control levels after 7 days of treatment, it could be expected that protein levels might not return to control levels either.

Previous studies have revealed that the expression of secretory proteins which are common to different tissues can be variably affected in diabetes. The labeling density of secretory granules for amylase is reduced by about 50% in the diabetic parotid gland (Szczepanski et al., 1998), whereas it is virtually abolished in the pancreas (Grégoire and Bendayan, 1986, 1987; Szczepanski et al., 1998). For secretory enzymes, Anderson and Shapiro (1980) found that peroxidase activity decreased in the diabetic submandibular gland, while it increased 54% in the parotid gland (Anderson et al., 1983). In this study, we observed that the granule labeling density of PRP in the submandibular gland is minimally reduced in diabetes, while Szczepanski et al. (1998) noted a significant decrease in the parotid gland. Our studies, however, noted that the granule labeling density of RII was minimally affected in diabetes in both glands. Insulin treatment had no effect on the restoration of PRP and RII to control levels in either gland. The fact that insulin did not restore the proteins to control levels suggests that other hormonal factors are also involved in regulating the PRP and RII genes. The differences in PRP expression between the submandibular and parotid gland in diabetes indicate that the effects of diabetes on submandibular PRP gene expression is relatively weak compared to the parotid gland. The differences could also indicate that the regulation of the PRP gene in each gland is tissue specific, and that different hormones are involved in its expression in the two salivary glands.
The RII protein is present not only in salivary gland acinar secretory granules (Mednieks et al., 1987; Szczepanski et al., 1998), but it is predominantly an ubiquitous cellular constituent that is evident in the acinar nuclei and cytoplasm and in other rat tissues such as the heart and brain (Szczepanski et al., 1998). Szczepanski et al. observed a significant decrease in RII labeling of the acinar nuclei in the diabetic parotid gland, while insulin treatment partially reversed the changes. In our study RII nuclear labeling was minimally affected in the diabetic submandibular gland, and significantly decreased in insulin-treated diabetic tissues compared to control tissues. This follows the same pattern we observed in RII labeling density of secretory granules.

Western blotting of the secretory proteins was used in our study for comparison to the quantitative immunocytochemistry results and to determine if whole proteins or their proteolytic fragments were detected by the antibodies. Although no statistical analysis was performed, similar patterns of secretory protein expression in Western blotting were seen compared to immunogold labeling. The protein concentration of mucin had a large decrease in the diabetic submandibular gland, while PRP and RII levels were minimally reduced. Subsequent insulin treatment, however, had a different effect on each protein. It restored the concentration of each protein to varying degrees compared to control levels. This contrasts with the results obtained with immunogold labeling, where no significant effect of insulin treatment on the restoration of the secretory proteins was observed.

Immunogold labeling and Western blotting measure protein expression in different ways which could account for discrepancies seen in the results for the insulin-treated diabetic tissues. In immunogold labeling, the proteins can be localized and quantitated in specific cellular compartments such as secretory granules. Western blotting, on the other hand, utilizes extracts of homogenized salivary gland tissue which contain proteins from all cellular compartments including secretory granules, endoplasmic reticulum, Golgi apparatus, and ductal lumina. Also, different epitopes are available on the PolyBed plastic compared to the nitrocellulose membranes that could affect binding of the primary
antibodies. Another factor to be considered is that the expression of the secretory proteins in the submandibular gland is small compared to the parotid gland which could affect their quantitation in Western blotting.

Western blotting of parotid secretory proteins was performed to confirm the results seen for the submandibular secretory proteins. The significant decrease seen in diabetic parotid PRP concentrations in Western blotting has been observed in previous studies. Based on changes in electrophoretic banding patterns, Anderson and Johnson (1981) and Anderson et al. (1991,1993) also found that diabetes decreases parotid PRP levels. The effect of insulin on PRP levels, however, was not examined in their study. We observed a partial restoration of PRP to control levels with insulin treatment.

The Western blotting results indicate that the major electrophoretic patterns of the three secretory proteins, mucin, PRP, and RII, were consistent with their established molecular weights. This indicates that large whole proteins rather than their proteolytic fragments were detected. Thus, changes seen in the expression of secretory proteins in diabetes are most likely due to effects on synthesis rather than degradation.

Mucin, PRP, and RII of the rat submandibular gland share many similarities with their human analogs. These proteins have many functions that probably are important for oral health in both species. Mucin contributes to the formation of a thin salivary film which covers all intraoral surfaces. This coating is thought to hydrate and lubricate the soft tissues of the mouth and contribute to the formation of the acquired enamel pellicle, which may serve as a permeability barrier and help protect the underlying hard tissue from demineralization by acids formed by tooth-borne flora. Mucin, along with other factors in saliva, helps modulate both the number and type of microorganisms that colonize the mouth by favoring attachment and subsequent proliferation of certain organisms while promoting the clearance of others (Tabak, 1995). The significant decrease seen in the expression of mucin in diabetes and the lack of its restoration with insulin treatment may have important implications for an individual’s oral health.
The largest protein family in human salivary secretions, PRP, are proposed to be involved in calcium binding, hydroxyapatite binding, formation of the acquired enamel pellicle, and agglutination of oral bacteria (Carlson, 1988). Since PRP and mucin share many similar functions, the relatively normal expression of PRP in diabetes may offset some of the detrimental effects on oral health normally associated with the disease.

The cyclic AMP receptor protein (cARP), RII, is present in the secretory granules, nuclei, and cytoplasm of salivary gland acinar cells. While the precise role of RII as a secretory protein is unknown, it is a critical component of the major intracellular pathway regulating secretion (Quissell et al., 1988, 1993) and gene expression (Flockhart and Corbin, 1982; Lohmann and Walter, 1984; Krebs, 1992; Lin et al., 1993; Ann et al., 1997) in salivary glands. Although the effects of diabetes and insulin on RII expression have not been previously determined, other components of the cyclic AMP system are known to be altered. Cyclic AMP phosphodiesterase activity is decreased in experimental diabetes (Solomon, 1975; Solomon et al., 1989), and insulin inhibits the activities of adenylate cyclase (Illiano and Cuatrecasas, 1972) and PKA (Walkenbach et al., 1978). The expression of the secretory proteins, mucin (Kousvelari and Tabak, 1991) and PRP (Carlson et al., 1991; Lin et al., 1993; Ann et al., 1997), are known to be regulated by cyclic AMP. Thus, changes in the activity and/or expression of specific components of the cyclic AMP signaling system, such as RII, during diabetes and subsequent insulin treatment may influence submandibular gland function.

The regulation of gene expression for the submandibular gland secretory proteins involves a complex interrelationship among insulin, the sympathetic nervous system, and possibly other hormones that is not fully understood. Beta-adrenergic regulation (cyclic AMP-mediated) of gene expression, specifically transcription, in the parotid and submandibular gland has been partially elucidated. Future studies should concentrate on how insulin, through its signaling pathways, regulates gene expression in the normal
physiologic and disease states of the salivary glands. Additionally, the complex interactions between insulin and cyclic AMP should be addressed.
Tables
Table 1. The effects of experimental diabetes and insulin treatment on serum glucose levels and body weight.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Glucose (g/dL)</th>
<th>Body Weight Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>134 ± 17.3 (4)*</td>
<td>----</td>
</tr>
<tr>
<td>Diabetic</td>
<td>483 ± 23.7 (3)</td>
<td>-21.5 ± 2.28 (7)a</td>
</tr>
<tr>
<td>Insulin</td>
<td>339 ± 35.5 (4)</td>
<td>+ 7.40 ± 1.08 (4)b</td>
</tr>
</tbody>
</table>

* Mean value ± standard error (number of animals).

a Reduction in weight from control to diabetic state (number of animals).

b Increase in weight from diabetic to insulin-treated state (number of animals).
Table 2. Morphometric analysis of immunogold labeling of submandibular gland acinar secretory granules for mucin and PRP (gold particles/μm²).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mucin</th>
<th>PRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.2 ± 1.00 (29)*</td>
<td>31.8 ± 3.42 (30)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8.85 ± 0.83 (10)*</td>
<td>23.0 ± 1.75 (10)</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>7.03 ± 0.44 (48)*</td>
<td>26.6 ± 1.42 (50)</td>
</tr>
</tbody>
</table>

* Mean value ± standard error (number of fields).

* Statistically different from control (p < 0.001).
Table 3. Morphometric analysis of immunogold labeling of submandibular gland acinar secretory granules and nuclei for RII (gold particles/μm²).

<table>
<thead>
<tr>
<th>Group</th>
<th>Secretory Granules</th>
<th>Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.3 ± 0.66 (30)*a</td>
<td>17.2 ± 1.19 (30)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8.32 ± 0.44 (10)</td>
<td>13.3 ± 0.40 (10)</td>
</tr>
<tr>
<td>Insulin -treated</td>
<td>6.80 ± 0.25 (50)*</td>
<td>11.9 ± 0.56 (50)*</td>
</tr>
</tbody>
</table>

* Mean value ± standard error (number of fields).

* Statistically different from control (p < 0.001).
Table 4. The effects of experimental diabetes and insulin treatment on submandibular gland protein expression measured from Western blots.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mucin</th>
<th>PRP</th>
<th>RII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>.105</td>
<td>.031</td>
<td>.052</td>
</tr>
<tr>
<td>Diabetic</td>
<td>.076</td>
<td>.024</td>
<td>.048</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>.093</td>
<td>.030</td>
<td>.068</td>
</tr>
</tbody>
</table>

The expression of the specific proteins (mucin, PRP, and RII) are shown as a ratio of the specific protein measured by Western blotting to the total proteins applied to the PAGE lane. The values are averages of two experiments.
Table 5. The effects of experimental diabetes and insulin treatment on parotid gland protein expression measured from Western blots.

<table>
<thead>
<tr>
<th>Group</th>
<th>PRP</th>
<th>RII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>.130</td>
<td>.293</td>
</tr>
<tr>
<td>Diabetic</td>
<td>.078</td>
<td>.224</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>.120</td>
<td>.201</td>
</tr>
</tbody>
</table>

The expression of the specific proteins (PRP and RII) are shown as a ratio of the specific protein measured by Western blotting to the total proteins applied to the PAGE lane. Mucin is not found in parotid extract.

Values are from experiment 1. Values for experiment 2 (not shown) were lower due to total protein loaded, but they had the same relationships between groups.
Figures
Key to Abbreviations

B  Basal lamina
ER  Rough endoplasmic reticulum
G  Golgi apparatus
IC  Intercellular canaliculi
L  Lumen
LD  Lipid
LY  Lysosome
M  Mitochondrion
MC  Myoepithelial cell process
N  Nucleus
SMG  Submandibular gland
SG  Secretory granules

Figure Legends

Figure 1. Electron micrographs of control rat submandibular gland.

(A.) Several seromucous acinar cells surround the lumen. The cells are similar in size and contain many electron-lucent secretory granules (x 5,900; scale bar= 2 μm).

(B.) High magnification of an acinar cell exhibiting secretory granules, rough endoplasmic reticulum, and a mitochondrion. Note the fine filamentous nature of the granule content (x 47,000; scale bar= 0.5 μm).

Figure 2. Electron micrographs of diabetic rat submandibular gland.

(A.) Portion of a submandibular gland showing 3 seromucous acinar cells. Several intercellular canaliculi are seen. Note the lipid accumulation in the basal cytoplasm and the lysosomal body (x 9,000; scale bar= 1 μm).
(B.) High magnification showing deposition of extra basal lamina material, including folds and multiple laminae. Rough endoplasmic reticulum and mitochondria are present in the acinar cells (x 22,000; scale bar= 1 μm).

(C.) High magnification displaying coalescence of secretory granules and pooling of their contents (*). Mitochondria and Golgi apparatus are also shown (x 22,000; scale bar= 1 μm).

(D.) High magnification showing a lysosome. Mitochondria and Golgi apparatus are also present (x 22,000; scale bar= 1 μm).

Figure 3. **Electron micrographs of insulin-treated diabetic rat submandibular gland.**

(A.) Portion of a submandibular gland showing 5 seromucous acinar cells. Several intercellular canaliculi are present. Note the relatively normal morphological features and the absence of lipid accumulation (x 4,700; scale bar= 2 μm).

(B.) High magnification of an intercellular area between acinar cells exhibiting a myoepithelial cell process. Secretory granules, rough endoplasmic reticulum, and mitochondria are also shown (x 29,000; scale bar= 1 μm).

Figure 4. **Immunogold labeling of acinar secretory granules with anti-SMG mucin antibody.**

(A.) Control, (B.) diabetic, (C.) insulin-treated diabetic rat. Note the decreased reactivity of granules in the diabetic and insulin-treated diabetic tissues. Granule content appears aggregated due to the omission of osmium tetroxide for fixation in immunogold studies (x 43,000; scale bar= 0.5 μm).
Figure 5. **Immunogold labeling of acinar secretory granules with anti-PRP antibody.**

(A.) Control, (B.) diabetic, (C.) insulin-treated diabetic rat. Fewer gold particles are seen in the granules of diabetic and insulin-treated tissue (x 43,000; scale bar= 0.5 µm).

Figure 6. **Immunogold labeling of acinar secretory granules with anti-RII antibody.**

(A.) Control, (B.) diabetic, (C.) insulin-treated diabetic rat. Very little difference in reactivity of granules is seen between the control and diabetic tissues. A greater decrease in reactivity is seen in the insulin-treated diabetic tissue (x 43,000; scale bar= 0.5 µm).

Figure 7. **Immunogold labeling of acinar nuclei with anti-RII antibody.**

(A.) Control, (B.) diabetic, (C.) insulin-treated diabetic rat. Very little difference in reactivity of nuclei is noted between the control and diabetic tissues. A greater decrease is observed in the insulin-treated diabetic tissue (x 43,000; scale bar= 0.5 µm).

Figure 8. **Western blot of anti-SMG mucin.**

Molecular weight standards (R) are shown in lane 1. Submandibular gland (SMG) extracts are shown in lanes 2-4. A mouse monoclonal antibody against mucin recognized a single band of approximately 114 kD. (C) control, (D) diabetic, (I) insulin-treated diabetic rats.

Figure 9. **Western blot of anti-PRP.**

Molecular weight standards (R) are shown in lane 1. Submandibular gland
(SMG) extracts and parotid gland (PAR) extracts are shown in lanes 2-4 and 5-7, respectively. A rabbit polyclonal antibody against PRP recognized several bands of approximately 25-35 kD. (C) control, (D) diabetic, (I) insulin-treated diabetic rats.

Figure 10. **Western blot of anti-RII.**
Molecular weight standards (R) are shown in lane 1. Submandibular gland (SMG) extracts and parotid gland (PAR) extracts are shown in lanes 2-4 and 5-7, respectively. A rabbit polyclonal antibody against RII recognized a single band of approximately 50 kD. Several isoforms, RIIa and Rfr, were also recognized. (C) control, (D) diabetic, (I) insulin-treated diabetic rats.

Figure 11. **Densitometric scans of anti-SMG mucin in submandibular gland extracts.**
(A.) control, (B.) diabetic, (C.) insulin-treated diabetic rats. Note the decrease in the integrated density of mucin in the diabetic group and its reconstitution nearly to control levels in the insulin-treated diabetic group.

Figure 12. **Densitometric scans of anti-PRP in submandibular gland extracts.**
(A.) control, (B.) diabetic, (C.) insulin-treated diabetic rats. Note the decrease in the integrated density of PRP in the diabetic group and its reconstitution nearly to control levels in the insulin-treated diabetic group.

The protein expression of PRP in diabetes, however, was minimally reduced (Table 4).
Figure 13. **Densitometric scans of anti-RII in submandibular gland extracts.**

(A.) control, (B.) diabetic, (C.) insulin-treated diabetic rats. Note the decrease in the integrated density of RII in the diabetic group and its reconstitution above control levels in the insulin-treated diabetic group. The protein expression of RII in diabetes, however, was minimally reduced (Table 4).

Figure 14. **Densitometric scans of anti-PRP in parotid gland extracts.**

(A.) control, (B.) diabetic, (C.) insulin-treated diabetic rats. Note the decrease in the integrated density of PRP in the diabetic group and its reconstitution nearly to control levels in the insulin-treated diabetic group.

Figure 15. **Densitometric scans of anti-RII in parotid gland extracts.**

(A.) control, (B.) diabetic, (C.) insulin-treated diabetic rats. Note the decrease in the integrated density of RII in the diabetic group and its further decrease in the insulin-treated diabetic group.
Figure 8.

Figure 9.

Figure 10.
Figure 11.
Figure 12.
Figure 13.
Figure 14.
Figure 15.
References


Scott JD, Stofko RE, McDonald JR, Comer JD, Vitalis EA, Mangili JA. Type II regulatory subunit dimerization determines the subcellular localization of the cAMP-dependent protein kinase. J. Biol. Chem. 265: 21561-21566, 1990.


