INSULIN SECRETION BY RAT LACRIMAL GLANDS: EFFECTS OF SYSTEMIC AND LOCAL VARIABLES

Running title: Insulin secretion in the tear film

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Abstract

To understand the secretory mechanisms and physiological role of insulin in the tear film, the present study examined: (1) the time-course of insulin secretion in the tear film under glucose intravenous stimulation, (2) the glucose- and carbachol-induced insulin secretion from isolated lacrimal gland (LG), (3) the effect of insulin on glucose consumption by the cornea, and (4) the expression of insulin, PDX-1, and glucose transport proteins (GLUT) in lacrimal gland (LG) tissue. The insulin level in the tear film of 8-week-old male Wistar rats increased from $0.6 \pm 0.45$ to $3.7 \pm 1.3$ ng/ml in the initial minutes after glucose stimulation. In vitro assays demonstrated that higher glucose concentrations from 2.8 to 16.7 mM, 200 \textmu M carbachol or 40 mM KCl significantly increased insulin secretion from lacrimal glands compared to controls, but did not detect C-peptide as measured by RIA. Glucose consumption by corneal tissue, evaluated by radiolabeled D-[U-$^{14}$C] glucose uptake, was $24.07 \pm 0.61$ and was enhanced to $31.63 \pm 3.15$ nmol/cornea/2 h in the presence of 6 nM insulin ($P=0.033$) and to $37.5 \pm 3.7$ nmol/cornea/2 h in the presence of 11.2 mM glucose ($P=0.015$). Insulin and PDX-1 mRNA was detected in LG. Insulin was located in the apical areas of acinar cells by immunoperoxidase and the expression of GLUT-1, but not PDX-1, was confirmed by Western blot. These findings suggest that insulin secretion in the tear film is influenced by local stimuli such as nutrient and neural inputs and that this hormone plays a metabolic role in ocular surface tissues. These data also indicate that under normal conditions the insulin secreted by LG is stored, but it is not clear that is locally produced in the LG.

**Key words**: insulin, lacrimal gland, GLUT, tear film, ocular surface.
Introduction

The tear film carries immunoglobulin, lysozyme and lactoferrin involved in ocular surface defence, growth factors responsible for cell proliferation and differentiation, and hormones, including insulin, that provide signals for various types of information to peripheral tissues (19, 26, 32, 41).

Insulin mediates nutrient influx, energy storage, gene expression, and DNA and protein synthesis for various tissues by a process based on binding to its tetrameric transmembrane-specific receptor (25). Insulin has been detected in other exocrine gland secretions like saliva and milk (7, 45) and its pleiotropic action has been identified in the lacrimal tissue and on the ocular surface as well (13, 42, 45). In addition, topical insulin therapy has been used to promote corneal wound healing and to treat diabetes mellitus (DM) (15, 28, 34, 47).

Recent studies have detected unique insulin activities related to the eye and visual pathways, such as orientation of axonal linkage between retina and visual cortex in the embryonic stage of life and regulation of aqueous humour flow during adult life (18, 38).

The role of insulin on the ocular surface is also inferred by tear film and corneal epithelial cell disorders in diabetic patients (9, 11, 14). From in vitro work, it is known that insulin is necessary for corneal and lacrimal gland cell culture (13, 20, 27). Some signaling elements that mediate the action of insulin on the lacrimal gland and ocular surface in various conditions have been recently studied (30-32). Other proteins under the influence of insulin signaling are the glucose transporter (GLUT) family. GLUTs are responsible for glucose transfer through the cell membrane’s lipid layer and may also be present in the LG (21, 40).
Classically, pancreatic islet beta cells are responsible for insulin production; however, various reports support the hypothesis of a capacity of insulin storage or local production in exocrine glands and other tissues in pathologic conditions, (24, 35) or in normal situations (1, 6-8, 33, 39, 45), although others do not accept this possibility, maintaining that some tissues actually have a unique capacity for insulin storage and concentration (29, 44).

The hypothesis of the present study is that insulin is a key element in the tear film and that its secretion is sensitive to systemic and local influence.

Our objective was to characterize insulin secretion in the rat tear film. More specifically, we wanted to investigate the effects of fasting, acute glucose injection and other secretagogues (i.e., KCl and Carbachol) on insulin secretion by the lacrimal gland, as well as to obtain a better understanding of the possibility of extra-pancreatic insulin production.
Material and Methods

Animals

Eight-week-old Male Wistar rats from the university’s Animal Breeding Center were fed standard rodent chow and water *ad libitum*. Food was withdrawn 12-14 h before the beginning of the experiments. All experimental procedures conformed to The Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals and were approved by the Research Ethics Committee of the institution.

Sample collection

Rats were anesthetized with sodium thiopental (Cristália, Itapira, SP, Brazil), 100 µg/kg of body weight injected IP. For the analysis of the effect of glucose on insulin secretion in the tear film, tears and blood samples were collected from 0 to 60 min after glucose injection (1g/kg body weight). Blood was collected from the caudal vein with heparinized hematocrit tubes. Tears were collected with graduated Pasteur pipettes from the ocular surface with minor manipulation and transferred to Eppendorf tubes containing 50 µl of 0.9% NaCl. The samples were frozen at -75º C until the time for use.

In vitro insulin secretion

Under anesthesia, the pancreas and LG were removed from the animals. The tissues were immersed in Krebs-bicarbonate buffer on separate Petri dishes for each experimental group and tissue.

The pancreatic tissue was digested with collagenase as described (4) and LG tissue was cut with fine scissors into fragments with a mean volume of 1 mm³ under a light microscope. LG samples consisted of groups of two LG fragments, that were first
incubated in cell strainers for 45 min at 37 °C in Krebs-bicarbonate buffer containing 5.6 mmol glucose/L and equilibrated with 95% O₂/5% CO₂, pH 7.4; samples of 5 islets (0.1 mm³) were run in parallel for comparison. The solution was then replaced with fresh Krebs-bicarbonate buffer and the islets were further incubated for 1 h in medium of the following composition: 2.8, 8.3 and 16.7 mM glucose, 16.7 mM glucose combined with 20 µg/ml diazoxide, 200 µM carbachol, and 200 µM carbachol combined with 66 µg/ml atropine or 40 mM potassium. The incubation medium contained 115 mM NaCl, 5 mM KCl, 24 mM NaHCO₃, 2.56 mM CaCl₂, 1 mM MgCl₂, and 3 g/l bovine serum albumin (BSA).

After the incubation period, the supernatant of each experimental preparation (n=5 per preparation) and negative controls were collected and processed by radioimmunoassay (RIA).

To measure the total content of insulin in LG and isolated islets, samples were homogenized with polytron PT1200C (Brinkmann Instruments, Westbury, NY, USA) in alcohol-acid solution (20% ethanol, 0.2 N HCl) and also processed by RIA.

**Insulin and C-peptide quantification**

The insulin content in the tears, plasma, supernatant of in vitro experiments and homogenized tissues was measured by RIA. The C-peptide content in homogenized LG and pancreatic islets was also evaluated by RIA.

To ensure sensitivity, specificity and reproducibility of the method, the following procedures were performed: (a) curves with triplicate samples of commercially available insulin and C-peptide (Amersham, Aylesbury, UK and Linco, St. Charles, MO, USA, respectively) were run in parallel, (b) samples with similar dilutions of IGF-1 (Sigma, St. Louis, MO, USA) or containing only buffer were also analyzed, and (c) assay samples were run in duplicate. Sensitivity ranged from 0.1 to 20
ng/ml for insulin and from 25 pM to 1.6 nM for C-peptide, and interassay and intra-assay coefficients of variation were estimated at 0.12 and 0.075 for insulin and 0.10 and 0.063 for C-peptide, respectively.

**Glucose metabolism**

Glucose oxidation was measured in isolated cornea samples as previously described (3). Corneas excised from euthanatized 8-week-old male Wistar rats were placed in wells containing Krebs-bicarbonate buffered medium (50 µl) supplemented with trace amounts of D-[U-\(^{14}\)C] glucose (10µCi/ml). Four experimental conditions were compared: (1) 5.6 mM glucose without insulin or (2) with 6.0 nM insulin, (3) 11.2 mM glucose, and (4) 5.6 mM glucose with 60 nM insulin, to complement the fixed amounts of radioactive glucose present in each sample (n=5/group). The content of the wells was suspended in 20 ml scintillation vials which were gassed with 5% O\(_2\) and 95% CO\(_2\) and capped airtight with a rubber membrane. The vials were shaken continuously for 2 h at 37\(^{\circ}\)C in a water bath. After incubation, 0.1 ml 0.2 N HCl and 0.2 ml hyamine hydroxide were injected through the rubber cap into the glass cup containing the incubation medium and into the counting vial, respectively, to stop the oxidative reaction. After 1 h at room temperature, the internal flask containing the tissue and the solutions was separated, 6 ml of scintillation fluid was added to the external flask and the radioactivity present in \(^{14}\)CO\(_2\) molecules resulting from glucose oxidation was counted. Glucose utilization was measured by the generation of \(^{14}\)CO\(_2\) in a scintillator analyzer, in parallel with identically treated blank incubations. The rate of glucose oxidation (i.e. production of \(^{14}\)CO\(_2\)) was directly proportional to the radioactivity counted in the flasks and is expressed as pmol/cornea/1 h.

**RT-PCR for insulin and PDX-1**
The reverse transcription polymerase chain reaction (RT-PCR) was used to identify insulin II (rats and mice have two insulin genes) and PDX-1 mRNA in rat LG. In addition, RPS-29 mRNA, a sequence that determines the ribosomal protein subunit 29 expressed in eukaryotic cells, was used as control. Total RNA was purified from LG and pancreatic islet samples (positive control) using Trizol (Invitrogen, San Diego, CA, USA) and treated with deoxyribonuclease I (Gibco/BRL) to eliminate DNA contamination. Samples of the resulting RNA were quantified by spectrophotometry at 260 nm and evaluated in 6.6% formaldehyde, 1 % agarose (Gibco/BRL) gels to ensure RNA integrity. Reverse transcriptase, oligo dT priming and the Advantage RT-for-PCR kit from Clontech Laboratories Inc. (Palo Alto, CA, USA) were used for cDNA transcription.

PCR amplification of cDNA was performed with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using 1.5 units of Taq DNA polymerase (Gibco/BRL), 0.3 mM each of dATP, dCTP, dGTP and dTTP (Invitrogen), PCR buffer (Tris -Hcl 60 mM, MgCl2 1.5 mM, NH4 SO4pH10 15 mM) (Invitrogen), and 10 mM of 5’ and 3’ primers corresponding to rat insulin 2, PDX-1 and RPS-29 cDNA. The primers corresponding to rat insulin 2 (Accession number: NM 019130, sense: 5’ TTG CAG TAG TTC TCC AGT T and antisense: 5’ ATT GTT CCA ACA TGG CCC TGT 3’), rat PDX-1 (Accession number: NM 022852, sense, 5’ AAC CGG AGG AGA ATA AGA GG and antisense 5’ GTT GTC CCG CTA CTA CGT TT 3’) and rat RPS-29 (Accession number: BC 058150, sense, 5’AGG CAA GAT GGG TCA CTA CGT TT 3’ and antisense 5’AGT CGA ATC ATC CAT TCA GGT CG 3’) were designed by reference to GeneBank sequences and synthesized by Life Technologies (Gaithersburg, MD, USA).
The PCR program for insulin, PDX-1 and RPS-29 mRNA involved the following cycle profile: 32 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 57°C, extension for 1.5 min at 72°C, and maximization of strand completion for 7 min at 72°C. Following amplification, the cDNA fragments were analyzed on 1 % agarose gels containing a 100 bp DNA molecular weight leader (Gibco/BRL) and post-stained with ethidium bromide to confirm the anticipated 340, 225 and 202 bp sizes for insulin, PDX-1 and RPS-29 products, respectively. Preliminary assays were performed to ensure products in the linear range. In all PCR procedures, positive and negative control cDNAs were run in parallel, but separate, tubes. Positive controls for insulin and PDX-1 mRNAs included cDNA prepared from rat pancreatic islets. Negative controls included samples without reverse transcriptase or without cDNA samples. The results were registered in Gel Doc (Bio-Rad).

**cDNA Sequencing**

In order to confirm that insulin and PDX-1 mRNA were obtained by RT-PCR, cDNA samples was cloned in vetor pGEM T-easy (Promega, Madison WI, USA) and PCR was carried out in a thermal cycler PTC-200 Peltier Thermal Cycler (Perkin Elmer, Boston MA, USA)) with an initial denaturation step at 95 °C for 3 min, subjected to 25 cycles of denaturation 95 °C for 10 sec, annealing 50 °C for 5 sec, elongation 60 °C for 4 min. The reactions included 11 µl of water milliQ, 2 µl of BigDye buffer, 3 µl 5X buffer, 1 µl of primer and 3 µl of each template. The sequencing was performed in 310 Genetic Analyser ABI Prism(Perkin Elmer, Boston , MA, USA). Sequence specificity for insulin and PDX-1 was evaluated by using BLAST analysis.
Immunohistochemistry

Exorbital lacrimal glands and pancreas were excised and fixed in Bouin solution for 24 h. Tissue samples were embedded in paraffin, cut into 6 μm sections and transferred to poly-L-lysine (Sigma) pre-coated glass slides (Perfecta, São Paulo, SP, Brazil). The slides were incubated in 0.1% H₂O₂ for 5 min, washed in PBS (0.05 M sodium phosphate, 0.15 M sodium chloride, pH 7.3) and exposed to 2% normal goat serum solution (Vector, Burlingame, CA, USA) for 20 min at 4°C. The sections were then overlaid with an aliquot of purified mouse monoclonal anti-insulin (Dako, Carpinteria, CA, USA) at a final concentration of 2 µg/ml, prepared using 10 µl of antibody stock solution (200 µg/ml) diluted in 990 µl of 0.3% BSA (Gibco BRL, Gaithersburg, MD, USA) in PBS, or negative control solutions. The controls included 0.1% BSA in PBS and pre-immune IgG (Sigma) and sections of pancreatic islets. Following incubation for 4 h with primary antibody in a humidified chamber at 4°C, the sections were washed in PBS and incubated with a biotinylated goat anti-rabbit IgG antibody (Vector). After incubation with the second antibody, sections were again washed in PBS and incubated with an avidin-biotin complex (Vector) for 30 min at 25°C, before being developed with a diaminobenzidine substrate kit (Vector).

For histological correlation, conventional hematoxylin (Sigma) counterstaining was performed on tissue sections and the slides were covered with Entellan (Merck, Darmstadt, Germany) and a coverslip. Photographic documentation was done using a Leica DMLS microscope at 100 and 400 times magnification and ASA 100 Kodak film.

Western blotting for GLUTs and PDX-1

The tissues were excised under anesthesia as described above and homogenized in 400 µL of solubilization buffer (10% Triton-X 100, 100 mM Tris, pH 7.4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium...
vanadate, 2 mM PSMF, and 0.1 mg of aprotinin/mL) for 30 sec using a polytron PT1200C (Brinkmann Instruments). The tissue extracts were centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was added to 18 µL of Laemmli sample buffer and boiled for 5 min prior to loading for 12% SDS-polyacrylamide gel electrophoresis in a Bio-Rad miniature lab gel apparatus (Mini-Protean, Bio-Rad, Hercules, CA, USA). The electrotransfer of proteins from the gel to nitrocellulose was performed for 2 h at 120 V using a Bio-Rad miniature transfer apparatus. Non-specific protein binding to nitrocellulose was reduced by preincubating the filter for 2 h at 22°C in blocking buffer (3% BSA, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated for 2 h at 22°C with polyclonal antibodies, anti-PDX-1 (Chemicon, Temecula, CA, USA), GLUT-1, GLUT-2 and GLUT-4 (Santa Cruz, Santa Cruz, CA, USA) diluted in blocking buffer and subsequently washed for 30 min in blocking buffer without BSA. The blots were then incubated with 2 µCi of [125I] protein A (30 µCi/µg) in 10 ml of blocking buffer for 1 h at 22°C and washed again as described above for 2 h. [125I] protein A bound to the antibodies was detected by autoradiography using preflashed Kodak film at –70°C for 24 – 60 h. Images of the developed autoradiographs were scanned with a ScanJet 5p scanner (Hewlett-Packard, Boise, Idaho, USA) into Adobe Photoshop 7.0 on a personal computer.

**Electron Microscopy**

For transmission electron microscopy (EM), LG and pancreas were removed, fixed in 2% glutaraldehyde and 2% paraformaldehyde (Ladd Research Industries, Burlington, VT, USA) in 0.1 M cacodylate buffer, pH 7.4, containing 0.05% CaCl₂ for 40 min at room temperature. Tissues were postfixed in 2% OsO₄ (EM Sciences, Hatfield, PA, USA) for 1 h at room temperature, rinsed in distilled water, dehydrated
through a graded ethanol series, rinsed in acetone, and embedded in Embed 812 (EM Sciences). Thin sections (80-90 nm) were cut with a diamond knife and stained for 10 min each in Reynolds´ lead citrate and 2% uranyl acetate. Sections were examined with a Phillips EN 208 Electron Microscope. After photographic documentation, negatives were scanned and stored as digital files.

Statistical analysis

Data are reported as mean ± SEM. Insulin concentrations were compared by the Mann-Whitney U test (Statview software, Abacus, Berkeley, CA, USA). Glucose consumption by corneal tissue was compared among different media by ANOVA and by Fisher PLSD. The level of significance was set at P<0.05 in all analyses.
Results

The concentration of insulin in the tear film was $0.6 \pm 0.45$ ng/ml after a 12-h fast and increased to $3.7 \pm 1.3$ ng/ml after 5 min of intravenous glucose injection, continuing to increase up to 15 min and then returning to basal levels 1 h after injection (n=10 rats) (Figure 1). The blood glucose levels determined during this period are presented in Figure 2.

Assays were conducted to determine whether LG responded with insulin secretion in a manner similar to that of pancreatic β-cells. Isolated LG samples were exposed for 1 h to media with increasing glucose levels and submitted to cholinergic (i.e., carbachol) or depolarizing (i.e., K+) stimulation. The experiments showed that mean insulin levels in LG samples rose 36% in response to 200 μM carbachol compared to basal conditions (8.3 mM glucose). Mean insulin levels rose 82% in LG samples exposed to 16.7 mM glucose compared to 8.3 mM glucose. Samples exposed to 40 mM K+ added to media containing 2.8 mM glucose showed supernatant insulin levels 166% higher than 2.8 mM. The addition of 66 μg/ml of atropine inhibited by 22% the effect of carbachol on insulin secretion. Similarly, 20 μg/ml of diazoxide reduced to 62.5 % the effect of 16.7 mM glucose (Figure 3).

Similar assays performed with pancreatic islets from rats revealed that increased glucose levels determined higher insulin levels in the supernatant, which were much higher than those obtained with LG. Carbachol (200μM) induced high insulin levels, but the highest stimulation was obtained with 16.7 mM glucose (Figure 4). In negative control samples, no insulin was detected by RIA.

Moreover, the insulin levels in the LG extracts were $17.6 \pm 2.8$ ng/sample. The mean level in samples containing 5 pancreatic islets (which represent a 10 times smaller volume) was $170 \pm 23.5$ ng/sample (mean ± SEM obtained for 16 samples/group).
These data indicate that when similar amounts of tissue were compared, LG were found to contain about 100 times less insulin than pancreatic islets.

C-Peptide was evaluated in LG and pancreatic islets in order to verify the possibility of local production of insulin in LG tissue. The RIA revealed that C-peptide concentration is $5.77 \pm 0.38$ nM in pancreatic islets but is undetectable in LG (n=3/tissue).

To evaluate the influence of insulin on glucose uptake and metabolism by rat corneal tissues, glucose oxidation was measured in isolated corneal samples in order to infer about the action of insulin in the tear film. Corneal metabolism was measured by comparing glucose utilization by rat corneal tissues (n=5 corneas/condition). In the basal condition (5.6 mM glucose), glucose consumption was $24.07 \pm 0.61$ nmol/cornea/2 h. In condition two, in which 6 nM insulin was added to the medium containing 5.6 mM glucose, glucose consumption was $31.63 \pm 3.15$ nmol/cornea/2 h, a 32% rise compared to control (P=0.033). In a third condition, in which the glucose concentration was doubled to 11.2 mM and the assays were conducted without insulin, glucose consumption was $37.5 \pm 3.7$ nmol/cornea/120 min, i.e., 56% rise compared to control (P=0.015), but not significantly higher than condition two (P=0.14). However, in a fourth condition, in the presence of basal glucose levels (5.6 mM) and 60 nM insulin, glucose consumption was $31.1 \pm 1.76$ nmol/cornea/120 min (n=5 corneas/condition), which was 29% higher than the basal situation (P=0.005) but similar to that observed in the presence of physiologic levels of insulin (condition two) (P=0.88), (Figure 5).

Evaluation by RT-PCR and agarose gel electrophoresis indicated that insulin and PDX-1 mRNA sequences were present in rat LG. However, the intensity of the bands
indicated that much lower amounts are expressed in this tissue in comparison with pancreatic islets. RPS-29 mRNA showed a similar expression in both tissues (Figure 6).

To establish that PDX-1 and insulin RT-PCR products represent their cDNA, the amplified cDNA fragments from lacrimal gland were purified and subject to DNA sequence analysis. The resulting sequences matched those in the GenBank database, thereby verifying the identity of these products (data not shown).

Immunohistochemical analysis indicated the presence of insulin on the apical side of acinar cells of the rat LG and also in epithelial cells and ductal cells (Figure 7A). Marked staining was also obtained in samples from pancreatic islets (Figure 7B), although no specific staining was found in negative control tissues (Figure 7C and D).

In order to investigate the mechanism of glucose internalization in the LG that allows insulin secretion in response to extra cellular glucose levels, we investigated the presence of GLUT proteins in LG tissue. LG tissues and liver samples were processed for Western blot analysis and searched for the presence of GLUT-1, -2 and –4. In these assays GLUT-2 and –4 were not detected (data not shown); however, GLUT-1 expression was observed in rat LG tissues (Figure 8).

EM assays revealed secretory granules of variable size, distribution and density in acinar cells. Most of the small, round and higher density secretory granules were accumulated in the apical area of the acinar cells (Figure 9, left). Comparison with pancreatic β-cells indicated some similarity to insulin secretory granules in terms of density and circular shape; however the later have a small diameter and are surrounded by a white area (Figure 9, right).
Discussion

Previous data have identified insulin in the tear film of rabbits and humans (26, 31). Our study provides information about the presence of insulin in the tear film and LG of normal rats as indicated by immunohistochemistry, with a mechanism of secretion possibly based on secretory granules.

The present findings indicate that fasting reduces insulin levels in the tear film and this phenomenon is reversed by intravenous glucose injection \textit{in vivo}, in agreement with our findings in human tears and with observations of a systemic influence on insulin release from salivary secretion in diabetic and non-diabetic humans (31, 22).

Higher glucose levels also enhance the concentration of insulin secreted by LG \textit{in vitro}. Similarly, the presence of the cholinergic agonist carbachol or the depolarizing action of K\textsuperscript{+} increased the levels of insulin secretion in a similar way but at much lower concentrations than observed in pancreatic islets and was reversed by specific inhibitors, such as atropine for carbachol and diazoxide as a glucose transport inhibitor (12). This information indicates that LG insulin secretion is directly sensitive to environmental and systemic conditions and may not just reflect the rise in blood insulin levels.

The role of insulin in the tear film may involve promotion of metabolic events and cell proliferation in the tissues related to the ocular surface, as addressed by previous experimental studies (34, 13, 27, 46, 47). These previous data indicate that insulin is an adjuvant in corneal epithelium replacement \textit{in vivo}.

The mechanism involved in the internal recognition of extra cellular glucose levels and of glucose uptake was previously attributed to GLUTs (40, 2). GLUT-1 was identified on the ocular surface but its expression in corneal wound repair in diabetic and normal rats was not changed (43). Our finding of limited enhancement of glucose metabolism by corneal tissues in response to insulin compared to higher levels of
extracellular glucose without insulin confirms that this hormone enhances the glucose metabolism by corneal tissues but is not crucial for glucose intake, as characteristically mediated by GLUT-1.

The present study also identified only GLUT-1 in LG, which may represent the major facilitatory glucose transporter in LG and other exocrine glands and may play a role in the glucose content of exocrine secretion. This finding confirms the predominance of this isoform in epithelial cells and adult ocular tissues and indicates that the input of glucose to these glands is independent of insulin (21).

The facilitatory transport of glucose mediated by GLUT-1 may explain the finding of a limited capacity to release insulin in response to higher levels of glucose in LG, which could be attributed to the saturation of glucose influx trough GLUT-1. Conversely, the capacity of islet β cells to respond to 16.7 mM glucose was much higher than their capacity to respond to carbachol or K+, which also indicates a specialization of this tissue in metabolic control, attributed in part to the presence of GLUT-2 (40).

The expression of insulin and PDX-1 mRNA, a transcription factor involved in pancreatic islet cell differentiation and insulin expression (23, 17), without the presence of C-peptide and PDX-1 protein in detectable levels, indicates that extrapancreatic insulin production, as previously reported in other tissues, including rat retina (6, 8, 16), may occur, but not in meaningful levels in LG or is inhibited in its further steps in physiologic conditions.

Moreover, the presence of insulin and PDX-1 mRNA in the LG does not ensure that the identified mRNA sequences will be used in the transcription process. Rather, it is possible to speculate that insulin and PDX-1 mRNA may work on the RNA and protein process and on the transport of linked genes, as indicated by other studies with
different gene products (5, 10, 37). For PDX-1 mRNA, two homologous sequences previously described (Accession numbers S76307 and S67435) but not assigned to any gene would match to our sequencing product and that may indicate that it is cross reacting with our PDX-1 sequence.

A recent study in which insulin and other β-cell transcripts were detected in extra pancreatic tissues of diabetic mice, revealed, however, a much fainter signal compared to normal β-cell samples and variable expression of these elements depending on the DM model (16). In addition, previous work described embryonic stem cells expressing insulin without typical beta-cells granules and negative for C-peptide (36).

Further studies would open perspectives to the understanding of regulation of insulin gene expression, and to clarify whether extra pancreatic insulin production up-regulated in other tissues would be helpful in DM (16, 36).

The role of insulin on the ocular surface also needs to be addressed in physiologic and pathologic conditions in order to determine whether DM or other conditions related to dry eye present with reduced insulin secretion in the tear film. In addition, topical replacement of this hormone may be helpful to attenuate ocular surface complications in these cases.

In conclusion, our experiments raise the possibility that insulin secretion in the tear film is systemically controlled and may be sensitive to local influences able to mobilize this hormone from LG acinar cells. These observations confirm the ubiquitous distribution and actions of insulin and provide evidence of an additional role for the LG in the maintenance of the ocular surface.

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References


Figure Legends

Figure 1. Time course of insulin levels in tears and serum after glucose (1g/kg body weight) stimulation in rats. Samples were collected from anaesthetized rats (n=3) at the times indicated and measured by RIA.

Figure 2. Time course of rat glucose blood levels after caudal venous injection of glucose (1g/kg body weight) (n=3).

Figure 3. Static insulin secretion by LG after glucose, K+ or carbachol stimulation for 1 h. Insulin levels were measured by RIA in samples containing two fragments of LG tissues from Wistar rats. Samples were incubated under basal conditions (2.8 mM glucose) or stimulated with 8.3, 16.7 mM glucose, 20 µg/ml of diazoxide (Dzd) plus 16.7 mM glucose, 200 µM carbachol (CCh) plus 8.3 mM glucose, 66 µg/ml of atropine (Atr) plus 200 µM carbachol (CCh) and 8.3 mM glucose, or 40 mM KCl (K+) for 1 h. Data are representative of at least three independent experiments (n=4 samples/condition) and results are expressed as mean ± SE. (* indicates a significantly higher value than control; # indicates a significantly lower value than control, as indicated by the horizontal lines at the top).

Figure 4. Static insulin secretion by pancreatic islets after glucose, K+ or carbachol stimulation for 1 h. Insulin concentration (ng/ml) was measured by RIA in the supernatant of pancreatic islets incubated under basal conditions (2.8 mM glucose) or stimulated with 8.3, 16.7 mM glucose, 20 µg/ml of diazoxide (Dzd) plus 16.7 mM glucose, 200 µM carbachol (CCh) plus 8.3 mM glucose, 66 µg/ml of atropine (Atr) plus 200 µM carbachol (CCh) and 8.3 mM glucose, or 40 mM KCl (K+) for 1 h. Data are representative of at least three independent experiments (n=4 samples/condition) and results are expressed as mean ± SEM. (* indicates a significantly higher value than control; # indicates a significantly lower value than control n, as indicated by the
horizontal lines at the top).

Figure 5. Glucose metabolism in rat corneas exposed to 5.6 mM glucose, 5.6 mM glucose + 6.0 nM insulin, 11.2 nM glucose or 5.6 nM glucose + 60 nM insulin, evaluated by measurement of $^{14}$C consumption ($n=5$/group). (* indicates a significantly higher mean response compared to the basal situation (5.6 mM glucose), $P<0.05$).

Figure 6. Presence of PDX-1 and insulin mRNA in rat LG and pancreatic islets. RPS-29 mRNA was used as positive control. Samples were processed for RT-PCR, agarose gel electrophoresis and ethidium bromide staining. Photographs of agarose gels were obtained with a Polaroid camera and digitalized with a Hewlett-Packard scanner. The results are representative of two independent experiments.

Figure 7. Identification of insulin in RAT LG. The samples were processed for immunohistochemistry and developed with DAB. A: LG sample challenged with anti-insulin antibody, B: pancreatic islet with positive staining, C and D: LG and islet samples challenged with inactivated antibody (400x for LG and 100x for islets). The slides were counterstained with hematoxylin. The images are representative of three independent experiments.

Figure 8. Presence of GLUT-1 (A) and PDX-1(B) protein in rat LG. The numbers refer to the following samples: (1) Negative control (suppressed primary antibody), (2, 3): rat LG samples and liver (4-A) or pancreatic islets (4-B). Tissues were excised and homogenized in buffer A. After centrifugation, aliquots containing the same amounts of protein were run on 10% SDS-PAGE, transferred to nitrocellulose, and challenged with anti-GLUT-1 antibody ($\alpha$-GLUT-1) or anti-PDX-1 antibody ($\alpha$-PDX-1), followed by anti-mouse IgG, $^{[125]}$I protein A and subjected to autoradiography.
Figure 9. Electron microscopy of LG (left) and pancreatic islets (right). After extraction, tissues were fixed and processed for EM. LG acinar cells (left) presented a higher density of round shaped granules in the apical area (arrowhead) close to the acinar lumen (lu) and pancreatic islet β-cells (right) used for comparison presented abundant insulin secretory granules in the cytoplasm (arrowhead). Micrographs are presented at 1500 x magnification.
Figure 1

![Graph showing insulin secretion in the tear film and blood over time.](image1)

Figure 2

![Graph showing glucose levels over time.](image2)
Figure 4

The figure shows the insulin secretion (ng/ml) for different treatments: 2.8, 2.8K, 8.3, 8.3Cch, Cch+Atr, 16.7, and Dzd. The bars represent the mean insulin secretion, with error bars indicating the standard deviation. Symbols (*) and (#) denote statistical significance compared to control and other treatments, respectively.
Figure 5

- Glucose 5.6 mM
- Glu 5.6 mM + Ins 6 nM
- Glu 11.2 mM
- Glu 5.6 mM + Ins 60 nM

nmol/cornea/120 min
Figure 6
Figure 7
Figure 8

α-GLUT-1

46 kDa

1 2 3 4

α-PDX-1

45 kDa

1 2 3 4
Figure 9