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Impact of Gender on Insulin Signaling Pathway in Lacrimal and Salivary Glands of Rats

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The structure and function of lacrimal and salivary glands present gender differences. Previous works have indicated a synergic action between insulin and androgens over lacrimal gland, and insulin-signaling pathways were recently described in lacrimal gland and salivary gland. Our present study investigates whether gender modulates the early steps of the insulin-signaling system in vivo. Eight-week-old male and female Wistar rats (*n* = 8/group) were compared to evaluate insulin serum levels and insulin tolerance tests by radioimmunoassay and glucose oxidase method, respectively. To assess insulin receptor (IR), Shc, STAT-1, ERK, and Akt phosphorylation in response to insulin in lacrimal gland and salivary gland, tissues from female and male rats (*n* = 5–8/group) were submitted to immunoprecipitation and immunoblotting or Western blotting protocol, and phosphorylation level was determined by densitometry. No difference was found in insulin serum levels or insulin tolerance tests comparing both groups. Nevertheless, lacrimal gland and salivary gland of female rats had a significantly lower insulin-induced IR phosphorylation compared with males. IR phosphorylation was not affected by the estrous cycle stage in either tissue. In addition, in females an apparent but not significant lower STAT and Akt phosphorylation in response to insulin was observed in the lacrimal gland, compared with males. Our findings suggest that alterations in insulin signal transduction may play a role in lacrimal gland and salivary gland gender differences.

Key Words: Akt; ERK; lacrimal gland; insulin signaling; salivary gland; STAT-1.

Introduction

The endocrine system influences the structure, functional activity, and susceptibility to disease of lacrimal and salivary glands *(1,2)*. Some of the better-studied hormones known to exert these actions are the sex hormones and insulin; in addition, a synergic effect between those hormones in the lacrimal gland function has already been shown *(1–4)*.

Sex hormones, mainly androgens, are responsible for the morphologic, biochemical, immune, and secretory characteristics of lacrimal gland. They are also responsible for the gender differences observed in lacrimal and salivary glands of various species as well as play a major role in the etiology of Sjögren syndrome, a disease that causes dry eyes and dry mouth and is more prevalent in women *(1,5,6)*. Gender is also known to interfere in clinical aspects of both of the most common forms of diabetes mellitus, but the mechanisms are still not understood *(7)*. In addition, diabetes mellitus, a condition caused by impairment of insulin secretion and/or action, also presents with clinical signs of lacrimal gland and salivary gland dysfunctions as manifested by symptoms of dry eye and dry mouth *(8–11)*.

Insulin plays a major role in lacrimal gland and salivary gland functions as well. Previous studies have shown that reduced insulin availability lowers rat lacrimal tissue secretion and reduces lacrimal gland acinar cell growth in vitro *(3,4)*. Moreover, the ability of androgens to induce lacrimal acinar cell secretion and immune response was considerably reduced by insulin removal in live animals and culture cells *(3,4)*. Furthermore, insulin combined with testosterone significantly increased the weight of lacrimal gland in hypophysectomized rats, compared with controls treated with placebo or each hormone separately *(12)*.

Sex hormones may interfere with insulin action in other organs since increased circulating testosterone can improve insulin sensitivity in male but not in female rats *(13,14)*. Conversely, estrogen and progesterone may act as antagonists of insulin, as observed in pregnancy or postmenopausal hormone replacement therapy, and this may be owing to changes in the early steps of insulin signal transduction *(15, 16)*. This information, therefore, indicates a close interaction between insulin and sex hormones, which may be dose and organ specific and directly influenced by sex hormones on one or more steps of the insulin-signaling cascade. However, the molecular mechanisms responsible for the interaction between insulin and sex hormones in lacrimal gland remain to be elucidated.

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Table 1
Body Weight and Serum Insulin Levels in Males vs Females[a]

Group	*n*	Body weight (g)	Serum insulin (ng/mL)
Male	8	263.46 ± 8.26	0.987 ± 0.147
Female	8	175.62 ± 3.42*	0.870 ± 0.248

[a]Data are expressed as the mean ± SEM.
[b]$p < 0.05$ compared to the mean of male rats.

Table 2
K_{ITT} in Male and Females Rats[a]

Group	*n*	K_{ITT}
Male	8	4.25 ± 0.55
Female		
Estrus	5	3.46 ± 0.51
Diestrus 1	5	4.31 ± 0.34
Diestrus 2	5	3.75 ± 0.47
Proestrus	5	3.86 ± 0.54

[a]Data are expressed as the mean ± SEM.

Table 3
Densitometric Analysis of IR, Shc, and STAT-1 Proteins Following Antiphosphotyrosine Antibody Stripping and Membrane Probing with anti-IR, anti-Shc, and anti-STAT-1 Antibodies[a]

	Lacrimal gland			Salivary gland		
Group	IR	Shc	STAT	IR	Shc	STAT
Male	476.5 ± 217.1	270 ± 71.6	568.7 ± 175.5	437.7 ± 60.7	789.5 ± 129.2	265.7 ± 54.0
Female	605.8 ± 165.9	239.5 ± 59.05	575 ± 120.3	351.8 ± 87.5	907.3 ± 149.6	351.0 ± 190.5

[a]Data are expressed as the mean ± SEM ($n = 4$ for each group) in densitometric arbitrary units.

The action of insulin on target cells is triggered by the binding of the hormone to the insulin receptor (IR), followed by autophosphorylation at tyrosine residues. The activated and phosphorylated IR recruits and phosphorylates intracellular proteins such as IR substrates 1 and 2, critical for the mitogenic and metabolic effects of insulin *(17)*. Moreover, IR activation leads to the Shc adapter protein phosphorylation that further activates a signaling pathway that ultimately leads to the stimulation of a group of mitogen-activated protein kinases (MAPKs) called ERK 1 and ERK 2 *(18,19)*. Recently, the activation of the janus kinase signal transducer and activator of transcription (Jak-STAT) pathway in insulin signaling was described *(20,21)*. This pathway is also involved in cell proliferation *(22)*. Akt is an insulin-activated protein kinase that functions in the PI3 kinase pathway involved in cell survival *(23)*.

In recent studies, we were able to characterize the early steps of insulin signal transduction in lacrimal gland and salivary gland and to demonstrate tyrosine phosphorylation impairment in early elements of insulin-signaling cascade on lacrimal gland of diabetic rats *(24)*.

To begin to clarify the relationship among gender, sex hormones, and insulin action over lacrimal and salivary glands, we have analyzed elements involved in insulin signaling in these tissues of male and female rats.

Results

The basal ($t = 0$) glucose levels were 72.2 ± 4.7 mg/mL for females and 68.1 ± 4.7 mg/mL for males ($p = 0.29$). Male and female rats in the four estrous stages had similar K_{ITT} and blood insulin levels. In addition, male rats had significantly higher weight than females (Tables 1 and 2).

To determine the impact of gender on IR tyrosine phosphorylation, male and female rats were injected with insulin in the inferior cava vein and lacrimal gland and salivary gland tissues were collected 1.5 and 3 min later, respectively.

There was no difference in IR protein content in lacrimal gland and salivary gland of males compared with females (Table 3). Again, no difference was detected on IR basal level tyrosine phosphorylation in both tissues (Figs. 1A,E, and 2A). By contrast, scanning densitometry revealed an increase

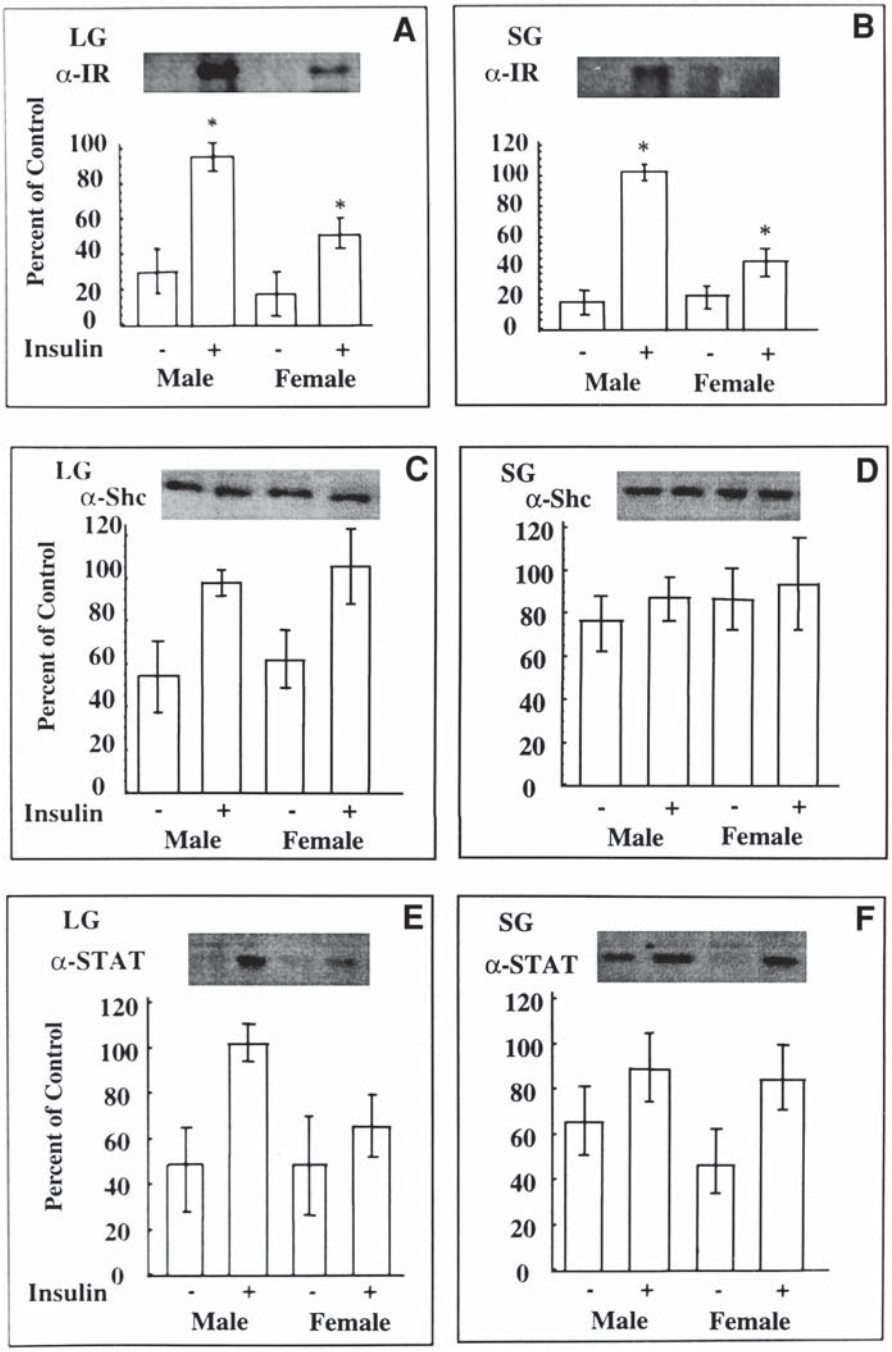

Fig. 1. Effect of gender on IR tyrosine phosphorylation (**A,B**), Shc tyrosine phosphorylation (**C,D**), and STAT-1 tyrosine phosphorylation (**E,F**) in rat lacrimal gland (A, C, and E) and salivary gland (B, D, and F). Lacrimal gland and salivary gland were excised 1.5 and 3.0 min, respectively, after insulin administration and homogenized in buffer A. After centrifugation, aliquots containing the same amounts of protein were run on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); transferred to nitrocellulose and detected with antiphosphotyrosine antibody (α-PY), followed by anti–mouse IgG, [^{125}I]protein A; and subjected to autoradiography. Values represent the mean ± SEM and are expressed as a percentage of the mean insulin-stimulated control (100%). *$p < 0.05$ compared to the mean control value. Results are representative of five experiments.

of 46.4 ± 4.47% in the insulin-stimulated IR tyrosine phosphorylation in lacrimal gland of male compared with female rats ($p = 0.009$). In addition, male rats had a 58.92 ± 6.59% increase on insulin-induced IR tyrosine phosphorylation in salivary gland, compared with females ($p = 0.0339$) (Fig. 1A,B).

To investigate whether reduced IR tyrosine phosphorylation propagates downstream the insulin-signaling cascade we decided to compare Shc, STAT-1, and ERK tyrosine phosphorylation and Akt serine phosphorylation in lacrimal gland and salivary gland of female and male rats, before and after insulin stimulation. No significant difference in

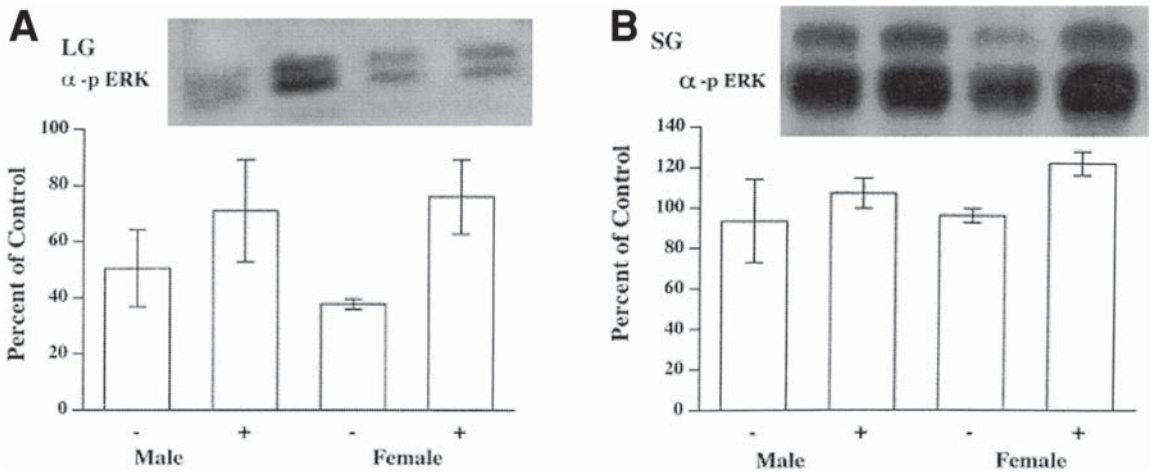

Fig. 2. Effect of gender on ERK tyrosine phosphorylation in rat lacrimal gland (**A**) and salivary gland (**B**). Lacrimal gland and salivary gland were excised 1.5 and 3.0 min, respectively, after insulin administration and homogenized in buffer A. After centrifugation, aliquots containing the same amounts of protein were run on 12% SDS-PAGE; transferred to nitrocellulose and detected with antiphospho ERK antibody (p ERK), followed by anti–mouse IgG, [^{125}I]protein A; and subjected to autoradiography. Values represent the mean ± SEM and are expressed as a percentage of the mean insulin-stimulated control (100%). Results are representative of three experiments.

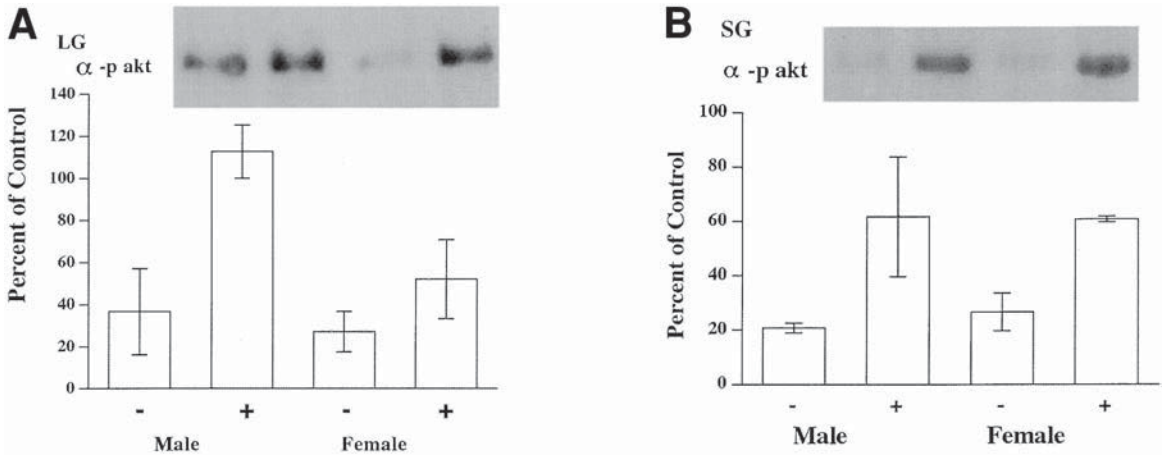

Fig. 3. Effect of gender on Akt serine phosphorylation in rat lacrimal gland (**A**) and salivary gland (**B**). Lacrimal gland and salivary gland were excised 1.5 and 3.0 min, respectively, after insulin administration and homogenized in buffer A. After centrifugation, aliquots containing the same amounts of protein were run on 8% SDS-PAGE; transferred to nitrocellulose and detected with antiphospho ERK antibody (p ERK), followed by anti–mouse IgG, [^{125}I]protein A; and subjected to autoradiography. Values represent the mean ± SEM and are expressed as a percentage of the mean insulin-stimulated control (100%). Results are representative of three experiments.

the quantity of Shc could be observed regarding gender or insulin injection (Table 3). In addition, either basal or insulin-induced Shc tyrosine phosphorylation was similar in lacrimal gland and salivary gland of female and male rats (Fig. 1C,D).

No significant difference could be detected in STAT-1 protein expression between the two groups (Table 3), and the basal levels of STAT-1 tyrosine phosphorylation were similar in lacrimal gland and salivary gland. There was a trend for 36.7% lower STAT-1 tyrosine phosphorylation in female lacrimal gland after insulin treatment compared with male (p = 0.06). No gender difference in STAT-1 tyrosine phosphorylation was detected in salivary gland after insulin stimulation (Fig. 1E,F).

The Western blotting assays revealed no difference in basal (p = 0.38) or insulin-stimulated (p = 0.72) levels of ERK tyrosine phosphorylation in lacrimal gland of both genders (Fig. 2A). In addition, the basal levels of ERK tyrosine phosphorylation were similar in salivary gland of male and female rats (p = 0.89) and remained comparable after insulin stimulation (p = 0.12) (Fig. 2B).

For Akt, again a trend for lower serine phosphorylation in response to insulin was observed in lacrimal gland of female rats compared with males (p = 0.055), despite a com-

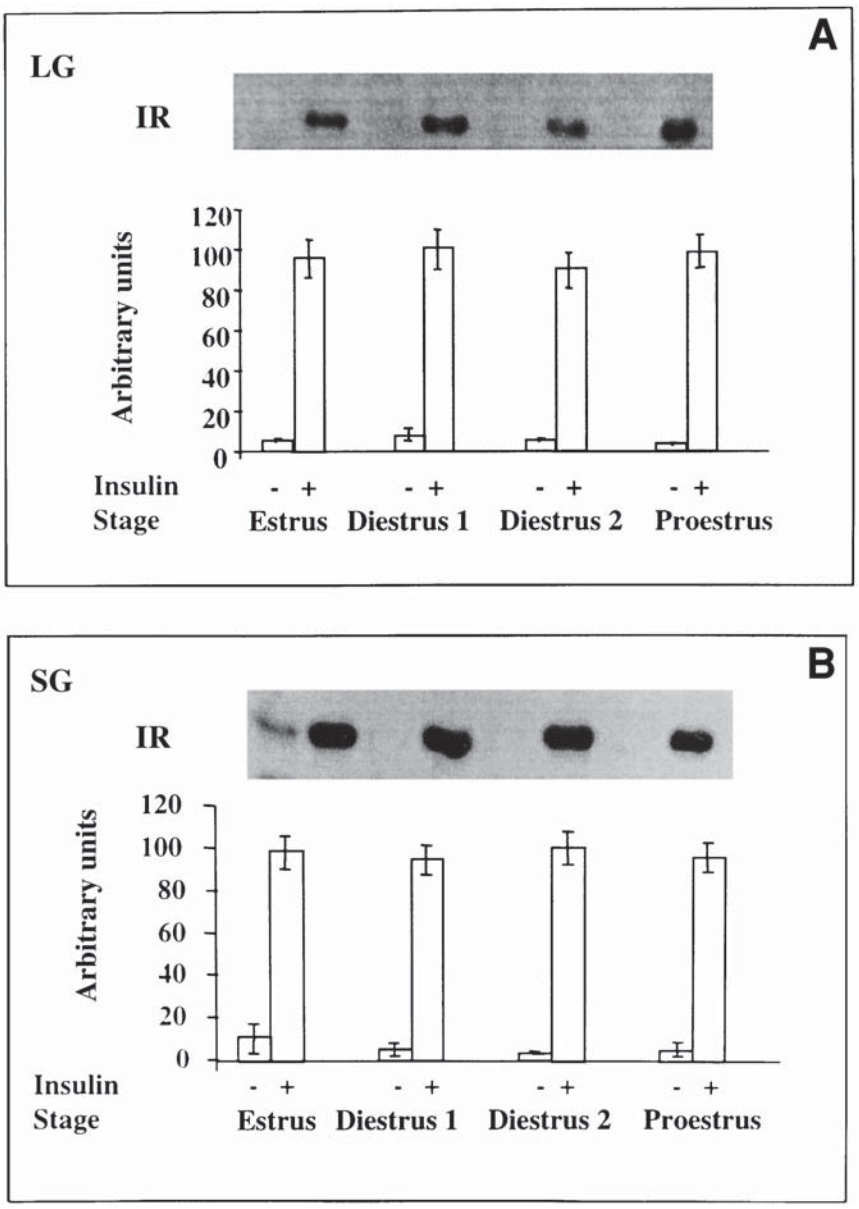

Fig. 4. Effect of estrous cycle on IR tyrosine phosphorylation in rat lacrimal gland (**A**) and salivary gland (**B**). Lacrimal gland and salivary gland were excised 1.5 and 3 min, respectively, after insulin administration and homogenized in buffer A. After centrifugation, aliquots containing the same amounts of protein were run on 8% SDS-PAGE; transferred to nitrocellulose and detected with antiphosphotyrosine antibody (α-PY), followed by anti–mouse IgG, [^{125}I]protein A; and subjected to autoradiography. Values represent the mean ± SEM and are expressed as a percentage of the mean insulin-stimulated control (100%). *$p < 0.05$ compared to the mean control value. Results are representative of three experiments.

parable basal situation in both genders ($p = 0.69$) (Fig. 3A). However, no difference was observed in Akt serine phosphorylation in salivary gland, in either basal or stimulated conditions ($p = 0.44$ and 0.96, respectively) (Fig. 3B).

To evaluate whether cyclical variations in female sex hormones were influencing the basal or insulin-stimulated IR activation, we investigated the IR phosphorylation in the four stages of the estrous cycle. Comparison of the IR phosphorylation in lacrimal gland and salivary gland at the four stages of the estrous cycle (estrus, diestrus 1, diestrus 2, and proestrus) demonstrated that no significant difference could be detected in lacrimal gland or salivary gland (Fig. 4A,B).

Discussion

Insulin, by acting through its receptor, promotes glucose uptake, storage of energy, gene expression, and protein synthesis *(17)*. A modulatory role for insulin on cellular signaling pathways of sex hormones or vice versa has been evaluated in insulin-sensitive tissues, but its importance on the physiologic behavior of such cells is not fully understood *(14,16)*.

Molecular crosstalk between the signaling pathways of different hormones or hormones, growth factors, and cytokines is currently a well-known phenomenon and may play a role in the fine control of cellular and tissue function *(25–28)*. These cellular events may contribute to clinical situations such as insulin resistance observed in infections or the common association between diabetes and hypertension *(29, 30)*. Related to a possible interaction between insulin and sex hormones, there are observations that women with type 2 diabetes mellitus have more complaints of dry mouth and dry eye than men *(31)*. In addition, some histologic and functional changes in lacrimal gland and salivary gland of nonobese diabetic mice were more intense in males than in females, unlike other autoimmune mice models used for studies of Sjögren syndrome *(6,32)*.

Our results demonstrate that in both lacrimal gland and salivary gland, IR phosphorylation is reduced in female compared with male rats. It is well known that lacrimal gland is a target organ for androgens in males and females *(2)*. The possibility that sex hormones may interfere with insulin signal transduction has been suggested to explain their negative effects on insulin sensitivity and may play a role in the events we have summarized *(14,16,33,34)*.

Because the observed reduction of insulin-induced phosphorylation of IR in females may influence the activity of distal elements of the signaling cascade, we decided to study the response of Shc, STAT-1, ERK, and Akt to insulin in male and female exocrine glands. Previous studies using cultured cells implicate Shc in prevention of apoptosis and cell proliferation *(18,35)*. In addition, Shc phosphorylation induced by insulin in liver and muscle of dexamethasone-treated rats is higher than in their placebo-treated controls *(36)*. Indeed, in breast cancer–cultured cells it was demonstrated that estradiol activates Shc and other peptides in the MAPK signaling pathway, in a mechanism that involve its classic receptor activation, which may suggest an influence of gender or sex hormones on Shc activation and cell proliferation *(37)*.

Similarly, studies of STATs' biologic actions indicate that they are involved in cell proliferation and differentiation, depending on ligands or biologic systems. Again, whether those signal transducers of various hormones, cytokines, and growth factors play any role in lacrimal gland or salivary gland dysfunction was not determined *(22,38)*. Interestingly, the epidermal growth factor, which shares various signaling pathways with insulin, has a well-characterized crosstalk with progesterone, involving Shc and STAT, suggesting a synergistic mechanism in mammary gland cells that may play a role in the growth of breast cancer *(39,40)*.

In the present study we were unable to identify any significant difference in Shc, STAT-1, ERK, or Akt phosphorylation related to gender in both tissues, despite a trend of decreased STAT-1 and Akt phosphorylation in response to insulin in female lacrimal gland. Our findings do not discard an influence of sex hormones over these signaling elements in lacrimal gland and salivary gland. However, they suggest that gender influence on IR phosphorylation does not propagate downstream unaltered in the pathways analyzed in this report. In the future, it would be worthwhile to evaluate other signaling elements that may connect to steroid receptor and insulin signaling.

The influence of the menstrual cycle on insulin action has been investigated in humans with contradictory data. This may be explained by failure to control for other metabolic parameters and variable sensitivity to measure insulin action *(41–43)*. In the present study neither IR phosphorylation in lacrimal gland and salivary gland nor K_{ITT} has shown significant differences when comparing the different estrous cycle stage of female rats. These findings may suggest that androgens rather than estrogen/progesterone might play a role in the gender difference of IR activation in lacrimal gland and salivary gland.

Since IR, Shc, and STAT-1 expressions were similar in the lacrimal gland and salivary gland of female and male rats, it is unlikely that sex hormones modulate the pattern of protein expression. Other mechanisms that shall be investigated include enhanced activity of phosphotyrosine phosphatases and interference with insulin-receptor interaction or with its intrinsic kinase activity.

In summary, our study shows that IR activation is decreased in lacrimal gland and salivary gland of female compared with male rats. These findings might be related to local interaction with sex hormones, mostly androgens. Considering the diverse physiologic effects of insulin, it may help to explain the gender differences in those tissue dysfunctions. Further investigation is necessary to clarify the molecular modulator mechanisms of sex hormones over IR and the additional impact on the insulin-signaling system.

Materials and Methods

Animals

Male and female 8-wk-old Wistar rats provided by the university's Animal Breeding Center were fed standard rodent chow and water ad libitum. Food was withdrawn 12–14 h before the experiments began. The Ethics Committee of the State University of Campinas (UNICAMP) approved all of the animal experiments, and the protocols involving animals followed the "Guidelines on the Handling and Training of Laboratory Animals" published by the Universities Federation for Animal Welfare.

Determination of Estrous Cycle

Eight-wk-old female rats ($n = 20$) were flushed with 0.9% NaCl by means of a micropipet in the vaginal orifice, samples were aspirated and placed on microscope slides, and the smear was examined under an optical microscope (Leica DMLS) every morning for 9 d, for estrous cycle stage determination and classification in estrus, diestrus 1, diestrus 2, and proestrus as previously described *(44,45)*.

Insulin Tolerance Test and Determination of Blood Insulin

Male and female rats were anesthetized with ip injection of sodium thiopental (Cristália, Itapira, SP, Brazil), at 100 µg/kg of body wt and used 10–15 min later, as soon as anesthesia was ensured by loss of foot and corneal reflexes. Blood samples for basal insulin level and iv insulin tolerance test (ITT) were collected from a caudal vein. Samples were collected at 0 (basal glucose and insulin determination), 4, 8, 12, and 16 min after injecting 10 µ*M* insulin intravenously. The samples were analyzed using a glucose oxidase method (Labtest, Lagoa Santa, MG, Brazil) and a standard radioimmunoassay method, respectively. The sensitivity range was between 0.1 ng/mL and 10 µg/mL, and inter- and intraassay coefficients of variation were estimated as 0.11 and 0.082, respectively.

The glucose plasma levels obtained from both groups during the 16-min interval after insulin injection were used to calculate plasma glucose disappearance (K_{ITT}) applying the formula $0.693/t^{1/2}$, as previously described *(46)*. In summary, plasma glucose $t^{1/2}$ was calculated from the slope of the least square analyses of the plasma glucose concentrations during the linear phase of decline.

Tissue Collection and Protein Extraction for Immunoprecipitation

For tissue collection, the abdominal cavity was opened, the inferior cava vein exposed, and 150 µL of insulin (10 µ*M*) (Biobras, Montes Claros, MG, Brazil) injected. This dose ensures a plasma insulin level approx 100 times higher than the postprandial level in adult rats and is able to induce phosphorylation of the elements under investigation as previously described *(16)*.

Samples of lacrimal gland (exorbital) and salivary gland (submandibular) were collected 1.5 and 3 min after insulin infusion, respectively. The tissues were coarsely minced and immediately homogenized in approx 10 vol of solubilization buffer A at 4ºC, using a Polytron PTA 20S homogenizer (model PT 10/35; Brinkmann). Buffer A (for solubilization) consisted of 1% Triton X-100, 50 m*M* HEPES (pH 7.4), 100 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride, 10 m*M* EDTA, 10 m*M* sodium vanadate, 2 m*M* phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), and 0.1 mg of aprotinin/mL (Bayer, São Paulo, SP, Brazil).

The extracts were centrifuged at 30,000*g* in an Eppendorf rotor at 4ºC for 20 min to remove insoluble material. Protein quantification was performed using the biuret dye method (Labtest), and equal amounts of protein (3 mg) were used for immunoprecipitation with 10 µL of the following antibodies: anti-IR, anti-Shc, and anti-STAT-1 rabbit polyclonal antibodies (200 µg/mL) (Santa Cruz Biotechnology, Santa Cruz, CA), as indicated, for a minimum period of 6 h at 4ºC with gentle agitation. The immune complexes were precipitated with 50 µL of protein A-Sepharose 6MB (Pharmacia, Uppsala, Sweden) under the same conditions for at least 2 h and washed three times with 50 m*M* Tris (pH 7.4) containing 2 m*M* sodium vanadate and 0.1% Triton X-100.

Immunoblotting

After washing, immunocomplexes obtained as described were resuspended in Laemmli sample buffer *(47)* containing 100 m*M* dithiothreitol and heated in a boiling water bath for 5 min. The samples were subjected to SDS-PAGE (8 or 10% Tris-acrylamide) in a Bio-Rad (Bio-Rad, Hercules, CA) miniature slab gel apparatus, in parallel with prestained protein standards.

Electrotransfer of proteins from the gel to Hybond ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) was performed for 2 h at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as previously described *(48)*, except for the addition of 0.02% SDS and β-mercaptoethanol (Bio-Rad) to the transfer buffer, to enhance the elution of high molecular mass proteins.

Nonspecific protein binding to the nitrocellulose was reduced by preincubating the membrane for 2 h at 22ºC in blocking buffer (5% nonfat dry milk, 10 m*M* Tris, 150 m*M* NaCl, and 0.02% Tween-20). The nitrocellulose filter was then incubated for 4 h at 22ºC with 25 µL of antiphosphotyrosine mouse monoclonal antibody (200 µg/mL) (Santa Cruz Biotechnology) diluted in 10 mL of blocking buffer (3% nonfat dry milk) and then washed three times (10 min each) in blocking buffer without milk. The blots were subsequently incubated with rabbit polyclonal anti–mouse IgG (1.25 mg) (Sigma) as a secondary antibody also diluted in 10 mL of blocking buffer with 3% nonfat dry milk for 2 h at 22°C, washed again three times (10 min each) in blocking buffer without milk, then incubated with 2 µCi of [^{125}I]-Protein A (30 µCi/µg) (Amersham, Aylesbury, UK) in 10 mL of blocking buffer (1% nonfat dry milk) for 3 h at 22ºC and washed again.

[^{125}I]-Protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at –70ºC for 12–72 h. Images of the developed autoradiographs were scanned (Hewlett Packard ScanJet 5p) into Corel Photo Paint 5.0 on a Compaq Presario, and band intensities were quantified by optical densitometry (NIH Image Analysis System). Preliminary assays were performed to ensure autoradiographic readings in the linear range.

The expression of signaling peptides was evaluated after membrane stripping, by incubating with anti-IR, anti-Shc, and anti-STAT-1 antibodies (6.6 µg) and reapplying the immunoblotting protocol described for antiphosphotyrosine antibody.

Western Blotting Protocol

Following a similar procedure for lacrimal gland and salivary gland extraction, tissue homogenization, and protein quantification in samples from male and female rats, a protocol to compare the expression of phosphorylated Akt

and ERK was performed, also with and without iv insulin stimulation.

Four hundred microliters of each sample was incubated with 100 µL of Laemmli buffer, and the volume to obtain 150 µg of protein was loaded in 8 and 12% SDS-PAGE and transferred to nitrocellulose membranes as described earlier.

After transfer and membrane blocking, 10 µL of antibody antiphospho Akt (Cell Signaling Technology, Beverly, MA) and 12 µL of antibody antiphospho ERK (Santa Cruz Biotechnology) were added to the respective membranes. Then the protocol for protein detection followed the same steps described earlier.

Statistical Analyses

Comparisons of the autoradiographs between female and male samples were done for each tissue (i.e., lacrimal gland or salivary gland) and each signaling element separately, using the Mann-Whitney U test and comparison among the samples of females in the four estrous cycle stages using analysis of variance (ANOVA) (Statview software, Abacus). Densitometric values are expressed as a percentage of the values for the first insulin-stimulated male samples (100%) present in each autoradiograph of the set of experiments comparing gender and for the first insulin-stimulated estrous-phase sample for the estrous cycle set of experiments (three to five per signaling element). The level of significance used was $p < 0.05$. Comparisons of blood insulin levels and K_{ITT} were done using student's *t*-test and ANOVA when applicable (Statview software).

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