Nuclear factor- κ B and advanced glycation end-products expression in lacrimal glands of aging rats

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Abstract

Advanced glycation end products (AGEs) increase with aging and induce signaling alterations that lead to inflammation and dysfunction in several tissues. Aging reduces function and insulin signaling in lacrimal glands (LGs). To evaluate whether AGE signaling and insulin secretion in LGs are altered in aging, 24- and 2-month-old male Wistar rats were compared. Immunohistochemistry with confocal microscopy was used to evaluate AGE, AGE receptor (RAGE) and nuclear factor- κ B (NF- κ B) expression in LGs. Basal tear secretion volume, insulin, interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) levels in tears and LGs and peroxidase activity in LG tissue were measured. Insulin secretion from isolated LGs and pancreatic β -cells was compared in the supernatant of aging and control rats *in vitro* by RIA after stimulation with 2.8–16.7 mM glucose, carbachol and

KCl. AGE, RAGE and NF- κ B expression was higher in LGs of aging compared with young rats. Basal tear secretion and peroxidase activity were significantly lower in the aging group ($P=0.016$ for both assays). IL-1 β and TNF- α levels were higher in tears of aging rats compared with young rats ($P=0.007$ and 0.05 respectively); however, even though aging rats were insulin-resistant (as confirmed by the insulin-tolerance test), the insulin levels in the tear film of aging and control rats were similar *in vivo* and *in vitro*. The higher expression of AGEs, RAGE and NF- κ B in LGs of aging rats is accompanied by systemic insulin resistance and may be involved in LG and tear film alterations but does not affect insulin secretion in the tear film. These observations indicate that metabolic events may be related to LG and tear film dysfunctions in aging.

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Introduction

It has been demonstrated that the process of aging is accompanied by morphological and secretory changes in lacrimal glands (LGs) that correlate with manifestations of dry eye (McGill *et al.* 1984, Bromberg & Welch 1985, Mathers *et al.* 1996, Schein *et al.* 1997, Draper *et al.* 1999, 2003). Among the pathophysiological mechanisms that underlie these changes, two possible events are the impairment of the mechanisms of insulin signal transduction (Carvalho *et al.* 1996, Rocha *et al.* 2003) and the formation of advanced glycation end products (AGEs), as also observed in other tissues (Vlassara *et al.* 1994, Stitt 2001). Taken together, these two hypotheses may predict that metabolic events, like insulin resistance, induce AGE accumulation in aging tissues and signaling alterations, leading to dysfunction in the secretory mechanisms of LGs.

Metabolic alterations involving early elements of the signaling cascades may be related to insulin reduction or

resistance and consequent organ dysfunctions in old rats (Castro *et al.* 1993, Perfetti *et al.* 1995, Paez-Espinosa *et al.* 1999, Rocha *et al.* 2004). One of the major consequences of insulin resistance, as observed in the elderly, is chronic hyperglycemia and oxidative stress leading to the formation of AGEs, obtained from chemical reaction of carbohydrates with proteins (Pongor *et al.* 1984, Singh *et al.* 2001, Evans *et al.* 2002). Previous studies have shown that AGEs alter the structure and functions of proteins, including those involved in cell signaling, and contribute to the chronic complications of aging (Vlassara *et al.* 1994, Zarina *et al.* 2000, Stitt 2001, Portero-Otin *et al.* 2002).

AGE binding to its receptor (RAGE) leads to the activation of the transcription factor nuclear factor- κ B (NF- κ B), which allows its translocation to the nucleus, where it regulates the expression of a large number of genes, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and RAGE (Singh *et al.* 2001, Evans *et al.* 2002). IL-1 β and TNF- α mRNAs are

expressed in LGs of animal models with exocrine gland inflammatory dysfunction and on the ocular surface of patients with dry eye syndrome, supporting the possible involvement of such cytokines in LG dysfunction in aging (Rocha *et al.* 1998, Solomon *et al.* 2001). In addition, IL-1 β and TNF- α are known to influence the function of LG tissues (Zoukhri *et al.* 2002). Therefore, the mechanisms by which hyperglycemia and oxidative stress act in age-related LG dysfunction might involve the formation of AGEs and activation of NF- κ B in the LGs, interfering with the signaling pathways and intracellular activity of cytokines such as IL-1 β and TNF- α .

Insulin is a powerful inotropic element for LGs in culture (Hann *et al.* 1991). It is present in the tear film, and insulin receptors (IRs) have been demonstrated in LGs (Hann *et al.* 1991, Rocha *et al.* 2000). In rats, insulin secretion from pancreatic islet cells (Castro *et al.* 1993, Perfetti *et al.* 1995) and the secretory response of LGs are impaired by aging (Draper *et al.* 2003). It is unknown whether insulin secretion is reduced in aging LGs following the secretory dysfunctions observed in these tissues.

In order to assess the involvement of insulin and AGE-related metabolic pathways in the pathogenesis of LG dysfunction in the elderly, the objectives of the present study were to identify the effects of aging on (i) LG expression of NF- κ B, AGEs and RAGEs, (ii) LG and tear content of the proinflammatory cytokines IL-1 β , and TNF- α , and (3) LG patterns of insulin secretion.

Material and Methods

Animal model

Male Wistar rats aged 2 months (young) and 24 months (aging) provided by the university's (UNICAMP) Animal Breeding Center received standard rodent chow and water *ad libitum*. Food was withdrawn 12–14 h before the experiments. All experimental procedures adhered to the *UFAW Handbook on the Care and Management of Laboratory Animals* and FRAME's guidelines and were approved by the Ethics Committee of the State University of Campinas (UNICAMP).

Immunohistochemistry

Exorbital lacrimal glands were excised from young and aging rats, embedded in Tissue Tek OCT (optimal cutting temperature) compound (Sakura Fine Tek, Torrance, CA, USA), frozen in liquid nitrogen and stored at -80°C . Tissue specimens were cut using a cryostat into 7 μm sections at -20°C and transferred to poly-L-lysine pre-coated glass slides (Perfecta, São Paulo, SP, Brazil). The slides were exposed to acetone for 10 min, washed in PBS (0.1 M sodium phosphate and 0.15 M NaCl, pH 7.4; Sigma Chemical Co., St Louis, MO, USA) and exposed to

3% BSA solution (Sigma) for 1 h at 22°C to reduce nonspecific protein binding. The sections were overlaid with rabbit polyclonal anti-NF- κ B subunit p65 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-AGE mouse monoclonal antibody (Research Diagnostic, Flanders, NJ, USA) or anti-RAGE rabbit polyclonal antibody (Santa Cruz Biotechnologies) at 4 $\mu\text{g}/\mu\text{l}$ concentration with 1% BSA in PBS. Following overnight incubation with primary antibody in a humidified chamber, the sections were washed in PBS and incubated for 2 h with anti-rabbit or anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (FITC; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a concentration of 375 $\mu\text{g}/\mu\text{l}$ in 1% BSA. After incubation with the secondary antibody, sections were again washed and counterstained with phalloidin (Sigma) concentrated to 0.1 $\mu\text{g}/\mu\text{l}$ for 45 min in a dark chamber. The slides were covered with Vectashield (Vector Laboratories, Burlingame, CA, USA) and a coverslip. Photographic documentation was performed using a confocal Zeiss Axiovert 200 M microscope (Zeiss, Wetzlar, Germany).

Determination of the expression of NF- κ B in nuclear and cytoplasmic extracts of LGs

Western blot studies to evaluate the expression of NF- κ B were performed on nuclear and cytoplasmic extracts of both study groups as described previously (Vaisse *et al.* 1996, Rocha *et al.* 2000). In summary, nuclear and cytoplasmic extracts were obtained from the exorbital LGs of young and aging rats ($n=6/\text{group}$ per experiment), collected under thiopental anaesthesia, and the tissues were coarsely minced and homogenized. The homogenate was centrifuged at 2000 g for 10 min at 4°C in an Eppendorf centrifuge 5804R, and the pellet was washed and resuspended in buffer A before being centrifuged again at the same speed for 10 min. The supernatant corresponded to the cytoplasmic extract. The pellet was then resuspended in a buffer B and then sonicated 10 times in a Potter-Elvehjem homogenizer (Kimble Kontes, Vineland, NJ, USA) with vigorous shaking for 30 min, and then centrifuged at 16 000 g for 30 min. The supernatant corresponded to the nuclear extract. The protein quantification was performed and equal amounts of protein (150 μg) were used for each sample in the Western blot studies. Samples were treated with Laemmli buffer and were subjected to SDS/PAGE (10% Tris/acrylamide) in a Bio-Rad miniature slab gel apparatus (Miniprotean; Bio-Rad Laboratories, Richmond, CA, USA), in parallel with pre-stained protein standards and β -mercaptoethanol (Bio-Rad, Hercules, CA, USA). Proteins were then electrotransferred from the gel to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, Little Chalfont, Bucks, UK) for 2 h at 120 V in a Bio-Rad miniature transfer apparatus (Miniprotean). After blocking, the membranes were then incubated overnight with

rabbit polyclonal anti-NF- κ B subunit p65 at a concentration of 0.4 μ g/ μ l in a buffer with 3% BSA, and then washed three times as described above. The blots were then incubated with 125 I-Protein A (Amersham Biosciences) and detected by autoradiography. Images of developed autoradiographs were scanned, and band intensities were quantified by optical densitometry using image-analysis software (Scion Image Analysis Software; Scion Corp, Frederick, MD, USA).

Determination of tear volume and of IL-1 β , TNF- α and insulin content in tears

Tear volume was measured in the animals of both study groups anaesthetized with an i.p. injection of sodium thiopental (100 μ g/kg; Cristália, Itapira, SP, Brazil) and used 10–15 min later, as soon as anaesthesia was assured by the loss of foot and corneal reflexes, using micropipettes (Eppendorf Research, Hamburg, Germany), and tears were collected and measured from the lacrimal meniscus, with an attempt made to collect the whole volume in one take and to avoid stimuli.

IL-1 β and TNF- α were measured in tears collected from aging and control rats with an ELISA kit according to the manufacturer's instructions (Pierce Endogen, Rockford, IL, USA). Tear samples from both eyes were pooled together.

For analysis of insulin secretion in tear film, tear samples were collected from rats fasted for 12 h. Tears were collected with graduated Pasteur pipettes from the ocular surface with minor manipulation, pooled from both eyes and transferred to Eppendorf tubes containing 50 μ l 0.9% NaCl. The samples were frozen at -75°C until the RIA, as described previously (Rocha *et al.* 2002a).

Plasma glucose disappearance test (K_{ITT})

To evaluate the plasma glucose disappearance rate in response to insulin injection, a test also known as the insulin-tolerance test, rats from both groups were anaesthetized with an i.p. injection of sodium thiopental, 100 μ g/kg body weight, and used 10–15 min later, as soon as anaesthesia was assured by loss of foot and corneal reflexes. Blood samples were collected from a caudal vein at 0 (basal glucose determination), 4, 8, 12 and 16 min after i.v. injection of 10 μ M insulin. The samples were analyzed by a glucose oxidase method (Labtest, Lagoa Santa, MG, Brazil). Plasma glucose disappearance rate (K_{ITT}) was calculated by the formula $0.693/t_{1/2}$. Plasma glucose half-life ($t_{1/2}$) was calculated from the slope of the least-squares analyses of the plasma glucose concentrations during the linear phase of decline, as described previously (Rocha *et al.* 2002b).

Peroxidase assay

To compare peroxidase activity in LG samples from aging and young rats, the tissues were exposed to a protocol

described previously (Stoppiglia *et al.* 2002). Briefly, samples were homogenized in Hanks solution at 4°C and the homogenates were centrifuged at 10 000 *g* for 8 min to remove cell membranes and organelles. The protein concentration of the supernatant was measured by the Biuret dye method (Labtest) and samples were collected and completed with Hanks solution to obtain 100 μ g/ml protein. H_2O_2 was added at a final concentration of 4.4 mM to each sample and controls were run in parallel. Aliquots were collected at 0, 5, 10, 20, 30, 40 and 60 min and peroxidase activity was measured by colorimetry (Glucose GODPAP, Laborlab, Brazil), against a H_2O_2 standard curve. Values were fitted with single exponential decay curves according to the formula:

$$[\text{H}_2\text{O}_2] \cdot (t) = [\text{H}_2\text{O}_2]_{t=0} \times e^{-kt}$$

The peroxidase activity of each sample is represented by the *k* value ($[\text{H}_2\text{O}_2]$ decay constant) obtained, expressed in $\mu\text{M}/\text{min}$.

In vitro insulin secretion

Under anaesthesia, the pancreas and LGs were removed from young and aging rats. The tissues were incubated in Krebs bicarbonate buffer on separate Petri dishes for each experimental group and tissue. The pancreatic tissue was digested with collagenase to allow islet isolation, as described by Boschero *et al.* (1995) and LG tissue was cut into fragments with a mean diameter of 1 mm under a microscope using fine scissors. 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 mM glucose and equilibrated with 95% $\text{O}_2/5\%$ CO_2 , pH 7.4. Samples of five islets 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 with media of the following composition: 2.8, 8.3 or 16.7 mM glucose, 16.7 mM glucose combined with 20 μ g/ml diazoxide, 200 mM carbachol, and 200 μ M carbachol combined with 66 μ g/ml atropine or 40 mM K^+ . The incubation medium contained 115 mM NaCl, 5 mM KCl, 24 mM NaHCO_3 , 2.56 mM CaCl_2 , 1 mM MgCl_2 and 3 g/l BSA.

After the incubation period, the supernatant of each condition ($n=5/\text{condition}$) and negative controls were collected and processed by RIA. To measure the total content of insulin in LGs and isolated islets, samples were homogenized with polytron PT1200C (Brinkmann Instruments, New York, NY, USA) in alcohol/acid solution (20% ethanol and 0.2 M HCl) and also processed for RIA.

Insulin quantification

The insulin content in tears, plasma, supernatant from *in vitro* experiments and homogenized tissues were

Figure 1 Representative confocal microphotographs demonstrating NF- κ B (p65 subunit) staining (green) in LGs of young (A) and aging (B) rats. The red is phalloidin counterstaining, indicating the localization of actin in the acinar or ductal cell membrane. The findings are representative of two independent experiments ($n=5$ /group). Scale bars, 20 μ m.

measured by RIA. To ensure sensitivity, specificity and reproducibility of the method, the following procedures were performed: (i) curves with triplicate samples of commercially available insulin (Amersham Biosciences) were run in parallel, (ii) samples with similar dilutions of insulin-like growth factor-1 (Sigma) or containing buffer only were also analyzed and (iii) assay samples were run in duplicate. The sensitivity range was 0.1–20 ng/ml, and the interassay and intraassay coefficients of variation were estimated at 0.11 and 0.076, respectively.

Statistical analysis

Data are reported as means $\pm$ S.E.M. Comparisons were made using the Mann–Whitney *U* test (Statview Software, Abacus, CA, USA), with the level of significance set at $P<0.05$.

Results

Immunohistochemical localization of NF- κ B, AGEs and RAGE

To locate and compare the expression of NF- κ B, AGEs and RAGE in LGs, samples from aging and control rats were processed in parallel and submitted to immunofluorescence by laser confocal microscopy ($n=5$ animals/group). Histological analysis demonstrated cytoplasmic and nuclear NF- κ B expression in acinar cells of LGs, with higher expression levels in nucleus and cytoplasm of LG acinar cells from aging animals compared with young on all slides examined (Fig. 1). AGE expression was observed in LG tissue samples, with more intense staining in the plasma membrane and cytoplasm of acinar cells and connective tissue of aging rats in all samples analyzed (Fig. 2). RAGE protein was also identified in LG tissues, predominantly with plasma membrane distribution in ductal cells and more intensely expressed in aging than in young rats (Fig. 3). RAGE expression in corneal

Figure 2 Representative confocal microphotographs demonstrating AGE staining (green) in LGs of young (A) and aging (B) rats. The findings are representative of two independent experiments ($n=5$ /group). Scale bars, 20 μ m.

epithelium and stroma was also more intense in aging animals than in young, as observed on all slides from both groups (Fig. 4).

NF- κ B expression in aging and young LGs

NF- κ B protein was detected in nuclear and cytoplasmic extracts of LGs, and higher levels of expression were

Figure 3 Representative confocal microphotographs demonstrating RAGE staining (green) in LGs of young (A) and aging (B) rats. The findings are representative of two independent experiments ($n=5$ /group). Scale bars, 20 μ m.

Figure 4 Representative confocal microphotographs demonstrating RAGE staining (green) in the cornea of control (left) and aging rats (right). The findings are representative of two independent experiments ($n=5$ /group). Scale bars, 50 μ m.

Figure 5 Effect of aging on NF- κ B p65 expression in cytoplasmic (A) and nuclear (B) extracts of LGs. Cytoplasmic and nuclear extracts ($n=6$ animals/group) were analyzed by Western blot using an anti-NF- κ B antibody specific for the p65 subunit. Values are means $\pm$ S.E.M. Values obtained for the young groups were defined as 100%, and values obtained for aging animals are expressed as a percentage of this value. Results are representative of two independent experiments. Higher expression of NF- κ B p65 was detected in nuclear and cytoplasmic extracts of aging animals ($P=0.02$).

detected in both nucleus and cytoplasmic extracts of aging animals compared with young ($n=6$ /group; $P=0.02$ in both cases). These results were confirmed in three independent experiments (Fig. 5).

LG weight, tear volume and peroxidase activity

Previous studies have demonstrated structural and functional changes in aging LGs (Bromberg & Welch 1985, Sullivan *et al.* 1990, Draper *et al.* 1999, 2003, Rocha *et al.* 2003). To ensure that this was the case in the present animal model, LG weight, tear volume and LG peroxidase activity were determined. Results are given in Table 1. The mean peroxidase activity levels were twice as high in young than in aging LGs ($P=0.016$; see Table 1 for details).

Table 1 Effect of aging on LG weight, body weight, basal tear volume, K_{ITT} , tear insulin level, IL-1 β and TNF- α levels and peroxidase activity of Wistar male rats. Means $\pm$ S.E.M. are shown

	Aging	Young
LG weight (mg)	142.60 $\pm$ 12.75*	77.20 $\pm$ 4.09
Body weight (g)	408.8 $\pm$ 18.17*	280.8 $\pm$ 4.65
Tear volume (μ l)	2.40 $\pm$ 0.25†	3.96 $\pm$ 0.42
K_{ITT}	3.15 $\pm$ 0.04†	4.65 $\pm$ 0.05
Tear insulin (ng/ml)	1.56 $\pm$ 0.38	0.83 $\pm$ 0.32
Tear IL-1 β (pg/ml)	6.22 $\pm$ 0.15*	4.26 $\pm$ 0.18
Tear TNF- α (pg/ml)	239.10 $\pm$ 50.02	57.32 $\pm$ 12.68
Peroxidase activity (μ M/min)	38.2 $\pm$ 6.7†	69.0 $\pm$ 15.0

* $P<0.05$, significantly higher than young; † $P<0.05$, significantly lower than young. $n=5$ for all parameters, except for tear volume where $n=10$.

Insulin, IL-1 β and TNF- α levels in the tear film

To investigate the metabolic and inflammatory consequences of aging for LGs, the content of insulin, IL-1 β and TNF- α was measured. The tear film insulin secretion in fasted rats was similar in both groups, with a tendency to higher levels in 24-month-old rats although, the difference was not statistically significant (Table 1). Both IL-1 β and TNF- α levels were significantly greater in aging rats ($P=0.007$ and 0.05 , respectively; Table 1).

In vitro insulin secretion

To determine whether LGs isolated from aging rats responded with insulin secretion in a similar way to young rats, assays were conducted with isolated LG samples exposed for 1 h to media containing secretagogues such as increased glucose levels (2.8–16.7 mM) and cholinergic (i.e. carbachol) or depolarizing (i.e. K^+) stimulation ($n=5$ /condition).

Mean insulin levels in the supernatant increased by 147% in medium containing 8.3 mM glucose compared with medium containing 2.8 mM glucose. Adding 200 μ M carbachol to the medium containing 8.3 mM glucose increased the mean level of insulin by 59%. Doubling the level of glucose in the medium from 8.3 to 16.7 mM increased the insulin level by 58%. The depolarizing effect of 40 mM KCl in medium containing 2.8 mM glucose increased the insulin levels by 61% compared with 2.8 mM glucose solely (Fig. 6). These experiments showed that insulin secretion in response to

Figure 6 Insulin release (ng/ml) from samples containing two fragments of LG tissues from aging (black bars) and young (grey bars) Wistar rats, incubated under basal conditions (2.8 mM glucose; 2.8) or stimulated with 8.3 or 16.7 mM glucose (8.3, 16.7), 200 μ M carbachol plus 8.3 mM glucose (8.3+Cch), or 40 mM KCl plus 2.8 mM glucose (2.8+K⁺) for 1 h. Data are representative of at least three independent experiments ($n=5$ samples/condition) and results are expressed as means $\pm$ S.E.M

higher glucose levels (2.8–16.7 mM) or to 200 μ M carbachol or 40 mM K⁺ behaved in a similar manner, with no statistical significance between groups.

Similar assays performed with pancreatic islets revealed that the insulin secretion response to low (2.8 mM) or physiologic (8.3 mM) levels of glucose was reduced in aged animals, but was slightly higher in response to higher levels of glucose (16.7 mM; $P=0.02$, 0.021 and 0.08, respectively), and was similar when stimulated with 200 μ M carbachol or 40 mM K⁺ (Fig. 7).

Comparison of total insulin content in LG tissue samples obtained from aging and young rats showed levels of 14.44 ± 2.80 and 16.06 ± 2.12 ng/ml, respectively ($P=0.81$). The mean level in pancreatic islet samples was

Figure 7 Insulin concentration (ng/ml) in the supernatant of pancreatic islets of aging (black bars) and young (grey bars) rats incubated under basal conditions (2.8 mM glucose) or stimulated with 8.3 and 16.7 mM glucose, 200 μ M carbachol plus 8.3 mM glucose, or 40 mM KCl plus 2.8 mM glucose for 1 h (column labels are as for Fig. 6). Data are representative of at least three independent experiments ($n=5$ samples/condition) and results are expressed as means $\pm$ S.E.M. * $P<0.05$, young versus aging rats.

1075.0 ± 106.10 and 1738.0 ± 150.0 ng/sample in the young and aging groups, respectively ($P=0.007$).

Discussion

The lower tear volume and peroxidase activity described here indicate a reduced secretory capacity with aging in rats, as reported previously (Bromberg & Welch 1985, Sullivan *et al.* 1990, Draper *et al.* 1999). In the present study, we demonstrated the presence of high levels of AGE and RAGE expression in LGs and RAGE in corneas of aging compared with young rats. Previous studies have reported that these mediators accumulate as a consequence of chronic hyperglycemia, are involved in widespread age-related pathology, and correlate with impaired hormone and growth factor signaling impairment, pro-inflammatory mediator recruitment and vascular damage (Vlassara *et al.* 1994, Stitt 2001, Portero-Otin *et al.* 2002).

In the present study, the mean LG weight of 24-month-old rats was significantly higher than in 2-month-old rats. This finding, apparently in conflict with a reduction in various functional parameters, may be explained by the acinar area replacement by connective tissue and reduction of metabolic, vascular and neural inputs over the secretory tissue. By 3 months of age, in spite of a stabilization, after a rapid increase in the LG weight there is a reduction in acinar density and the capacity to respond to cholinergic stimulation (Sullivan *et al.* 1990, Draper *et al.* 1999).

Although higher levels of NF- κ B expression were clearly demonstrated in the nuclear area of acinar cells in aging LGs, we could only demonstrate a trend towards higher levels of NF- κ B-induced downstream proinflammatory cytokines such as IL-1 β and TNF- α in the tear film of aged rats. This finding may be explained, at least in part, by the wide range of values obtained in the assays for these cytokines. Since inflammation has been linked previously to age-related LG dysfunction in humans, and the expression of AGEs and NF- κ B leads to an increase in the production of these proinflammatory cytokines, it is our opinion that further studies are necessary before the contribution of these inflammatory mediators to LG dysfunction in aging is definitely ruled out (Damato *et al.* 1984, Evans *et al.* 2002).

Insulin secretion by the LGs has been described previously, and is believed to be a mechanism by which this hormone is delivered to the ocular surface through the tear film (Rocha *et al.* 2002a). Therefore, our hypothesis was that LG dysfunction of aging would be associated with impairment of insulin secretion. In fact, old rats have higher insulin levels in pancreatic islets and blood, but they are heavier and have a reduced capacity for glucose storage, as observed here and elsewhere (Rocha *et al.* 2003). In a previous study, we have shown that aging is accompanied by reduced tyrosine kinase activity of the insulin receptor in LGs, an event that also occurs in liver, muscle and the central nervous system and may account in

part for the dysfunction of these tissue, indicating a lower capacity of insulin intake and secretion by exocrine glands (Carvalho *et al.* 1996). These phenomena may be explained by the reduced capacity of response of pancreatic β -cells and by the insulin resistance occurring in aging (Castro *et al.* 1993, Carvalho *et al.* 1996, Rocha *et al.* 2003, 2004). However, the similarity of insulin content and secretion by LGs in the aging and young groups may suggest mechanisms to preserve the levels offered to the LGs and ocular surface, and supports the theory that this hormone is a key element for LGs and ocular surface maintenance (Hann *et al.* 1991).

In conclusion, this study provides evidence for the hypothesis that the cumulative formation of AGEs and RAGEs, and the activation of NF- κ B, may be involved in the physiopathology of LG dysfunction of aging. Moreover, it confirms previous findings that metabolic alterations may lead to these dysfunctions, although insulin resistance does not impair insulin secretion by LGs in this model. Ongoing studies will help to elucidate whether any of these steps could be a target for therapeutic interventions that could avoid or reverse these events.

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