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Modulation of c-Jun NH_2-Terminal (JNK) by Cholinergic Autoantibodies from Patients with Sjögren's Syndrome

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ABSTRACT

***Background*:** *We wanted to determine* (*via an immunopharmacological approach*) *whether the c-Jun NH_2 terminal kinase* (*JNK*) *cascade is phosphorylated in the submandibular gland by carbachol and cholinergic autoantibodies* (*IgG*) *present in the sera of patients with primary Sjögren's syndrome* (*pSS*) *by interaction and activation of salivary gland muscarinic acetylcholine receptors* (*mAChRs*). ***Methods*:** *The JNK, PGE_2 and NOS assays were measured in rat submandibular gland with pSS IgG and carbachol alone or in the presence of different blocker agents.* ***Results*:** *pSS IgG-activated M_3 mAChRs stimulated JNK phosphorylation whereas the activation of M_1 mAChRs by carbachol stimulated JNK phosphorylation involving calcium-activated mechanism. The intracellular pathway leading to pSS IgG-induced biological effects on JNK activity involved activation of protein kinase C* (*PKC*), *inducible nitric oxide synthase* (*iNOS*) *and cyclooxygenase*-2 (*COX*-2) *enzymes. Also, activation of COX*-2 *and COX*-1 *by pSS IgG and carbachol-induced PGE_2 generation were involved. Conclusion*: *These results may contribute to better understanding the modulatory role of JNK enzymes by cholinergic autoantibodies from pSS patients acting on mAChR in rat submandibular gland.*

***Keywords*:** *JNK*, *pSS IgG*, *Autoantibodies*, *PGE_2*, *NOS*, *Carbachol*, *Cholinergic Antagonists*, *Enzymatic Inhibitors*

1. Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease of unknown etiology. Many autoantibodies have been reported to be present in pSS [1]. In particular; antibodies to the antigens SS-A/Ro and SS-B/La are commonly used in the diagnosis of pSS [2]. The presence of subtypes M_1 [3], M_3 [4,5] and M_4 [6] specific autoantibodies in 83% - 90% persons with pSS [7] is an important advance towards understanding the pathogenesis of pSS not only in terms of impaired glandular function, but also because of peripheral parasympathetic dysfunction associated with the disease [8,9].

IgG molecules from serum samples from patients with pSS have been reported to bind to glandular cholinoreceptors and act as partial agonists. IgG molecules have been reported to not only activate the receptor, but also to impair the response to the authentic cholinergic agonist [10], suggesting a defect in post-receptor signaling [11]. The most direct mechanism for preventing target organs from carrying out their function is that of early agonist-mediated activation of cholinoreceptors initiated by autoantibodies [12] which bind to, and persistently activate, cholinoreceptors [13]. Subsequently, the agonistic activity displayed by these autoantibodies may induce the desensitization [14], internalization and/or intracellular degradation of cholinoreceptors, leading to a progressive decrease in the expression and activity of these receptors [12].

Xerostomia and keratoconjunctivitis sicca result from lymphocyte infiltration of the salivary gland [15] and lachrymal gland [16]. The infiltrating cells interfere with glandular function by cell-mediated glandular destruction and production of autoantibodies that interfere with cholinoreceptors [17]. Dental caries resulting from the loss of salivary flow may be associated with periodontal disease [18].

Prostaglandins (PGs) are among the most relevant local mediators that participate in the modulation of acini cell functions under basal conditions [19]. PGs are re-

leased in large amounts during inflammation or in early stages of autoimmune diseases. In particular, overproduction of PGs has been shown to occur in neuroinflammatory diseases [20]. Also, nitric oxide (NO) plays a key part in the pathophysiology of systemic and chronic inflammatory disease and in the neurodegenerative process [21]. Recent evidence has indicated that there is constant crosstalk between NO and PGs biosynthesis pathways involved in the pathological mechanisms underlying certain inflammatory disorders [21,22].

In general, muscarinic acetylcholine receptor (mAChR) subtypes are grouped according to their functional coupling. This can be via mobilization of intracellular calcium (M_1, M_3. M_5) through the activation of phospholipase C (PLC), which results in the release of the second messenger inositol 1,4,5-triphosphate (IP_3) or by inhibition of adenylate cyclase (M_2, M_4), which results in reduction of the intracellular levels of cyclic adenosine monophosphate [23]. The same receptor may generate more than one set of intracellular second messengers and considerable crosstalk exists between signaling cascades [24]. The ability of these receptors to stimulate or inhibit cell growth has been attributed to differences in cell models, but the mechanisms involved in these cell type-dependent differences in growth response are unknown [25].

Mitogen-activated protein kinases (MAPKs) are activated by a diverse array of extracellular stimuli and regulate various cellular responses [26,27]. MAPK family members include c-Jun NH_2-terminal kinase (JNK) [28]. JNK is activated by cellular stress (ultraviolet and gamma radiation), osmotic and heat shock, inhibitors of protein synthesis, and inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1), but also weakly by growth factors (epidermal growth factor, EGF) [28,29]. JNK activation has been implicated in the immune response, oncogenic transformation, apoptosis [28, 30] and activation of two major transcription factors: activator protein 1 (AP-1) and nuclear factor-kappa B (NF-kB) [31,32]. In turn, AP-1 and NF-kB induce the transcription of several genes involved in acute and chronic inflammation as well as diseases of connective tissue [32]. The gene for the inducible isoform of nitric oxide synthase (iNOS) is involved in inflammation [32].

MAPK regulation by G protein-coupled receptors (GPCRs) appears to be a widespread phenomenon. It is also likely to mediate the proliferative and hypertrophic responses of cells to various hormones, neurotransmitters and local mediators that act at this class of receptor [33]. JNK activation has also been demonstrated for several GPCRs, including M_1 and M_2 mAChR [34-36], angiotensin [37], α_1-adrenergic [38], thrombin [39] and endothelin-1 [40]. Some studies support the notion of mobilization of intracellular calcium and activation of protein kinase C (PKC) [41] in cholinergic receptor-mediated JNK activation [35,37].

2. Aim

The aim of the present work was to examine the effect of cholinergic autoantibodies present in the sera of pSS patients and the authentic cholinergic agonist carbachol on JNK phosphorylation. We found that M_1 and M_3 mAChRs were coupled to JNK in the submandibular glands of rats. However, carbachol preferentially stimulated M_1 mAChRs whereas pSS IgG stimulated M_3 mAChRs. Both, the activation of M_3 and M_1 mAChRs by pSS IgG and carbachol stimulated JNK phosphorylation. The pSS IgG stimulation effect appeared to be mediated by activation of PKC, iNOS and cyclo-oxygenase-2 (COX-2) whereas the carbachol stimulatory effect on JNK was mainly associated with intracellular calcium-activated endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) with COX-1 stimulation. Also, the results indicated that in the JNK activation phenomenon, by pSS IgG and carbachol on salivary gland participated cholinergic M_3 and M_1 receptor, respectively. Our results suggested that activation of JNK by pSS IgG indicate that the enzyme may be involved in the pathological process of chronic sialodenitis present in the course of pSS.

3. Methodology

3.1. Drugs

Carbachol, pirenzepine, J104291, verapamil, calphostin C and thapsigargin were obtained from Sigma-Aldrich; FR-122047, DuP 697, methylisothiourea sulphate, L-NIO and NZ were from Tocris Cookson (Ellisville, MO, USA). Stock solutions were freshly prepared in the appropriate buffers. The drugs were diluted in a water bath to achieve the final concentrations stated in the text.

3.2. Animals

Male Wistar rats weighing 250 - 300 g from the Pharmacologic Bioterium (School of Dentistry, University of Buenos Aires) were used throughout. The animals housed in standard environmental conditions were fed with a commercial pellet diet and water *ad libitum*. For surgical removal of submandibular glands, the animals were sacrificed using ether. The experimental protocol followed the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23).

3.3. Subjects and Serological Tests

Females (age, 35 - 55 years) who had been diagnosed 7 - 15 y previously and who had been free of treatment for 8

months were selected from the metropolitan area of Buenos Aires. The study population was 25 women with pSS who presented with dry mouth, and 18 healthy women (mean age, 45 ± 10 years) without systemic disease (control group). The diagnosis of SS was based on four or more of the criteria published elsewhere [42]. Biopsy results, degree of xerostomia and keratoconjunctivitis sicca, and the results of serological tests in the different groups were the same as previously reported [10].

3.4. Peptides

A 25-mer peptide (K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I) and a 24-mer peptide (E-R-T-M-L-A-G-Q-C-Y-I-Q-F-L-S-Q-P-I-T-F-G-T-A-M) corresponding to the amino-acid sequence of the second extracellular loop of the human M_3 mAChRs and M_1 mAChRs, respectively, were synthesized from F-moc-amino acids activated using the 1-hydroxy benzo triazole/dicyclo hexyl carbodimide (HOBt/DCC) strategy and an automatic peptide synthesizer (Model 431A, Applied Biosystems, Foster City, CA, USA). The peptides were desalted, purified by high-performance liquid chromatography (HPLC), and subjected to amino-terminal sequence analysis using automatic Edman degradation and an Applied Biosystems 470A Sequencer. An unrelated 25-mer peptide (S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S) was synthesized as a negative control.

3.5. Purification of Human IgG

The serum IgG fraction from patients with pSS and from normal individuals (control) was isolated using protein G affinity chromatography as described elsewhere [9]. Briefly, sera were loaded onto the protein G affinity column (Sigma-Aldrich, St Louis, MO, USA) equilibrated with 1 M Tris-HCl (pH 8.0) and the columns were washed with 10 volumes of the same buffer. The IgG fraction was eluted with 100 mM glycine-HCl, pH 3.0, and immediately neutralized. The concentration and purification of IgG were determined using a radial immunodiffusion assay.

3.6. JNK Assay

Slices of submandibular glands of rats (20 mg) were incubated for 30 min in 500 μL of Krebs-Ringer bicarbonate (KRB) buffer and gassed with 5% CO_2 in O_2 at 37˚C. pSS IgG or carbachol were added 15 min before the end of the incubation period, and blockers added 15 min before the addition of different concentrations of pSS IgG or carbachol. The submandibular gland was then homogenized in 1.0 mL of cell lysis buffer (product 9803). We then followed the manufacturer instructions for the PathScan Total and Phospho-SAPK/JNK kit (Sandwich ELISA Kit; Cell Signalling Technology Incorporated, Beverly, MA, USA). JNK results were expressed as the optical density at 450 nm (OD 450 nm).

3.7. PGE2 Assay

Rat submandibular gland slices (20 mg) were incubated for 60 min in 500 μL of KRB and gassed with 5% CO_2 in O_2 at 37˚C. pSS IgG or carbachol were added 30 min before the end of the incubation period and blockers added 30 min before the addition of different concentrations of pSS IgG or carbachol. The submandibular gland was then homogenized in a 1.5-mL polypropylene microcentrifuge tube. We then followed the manufacturer instructions for the PGE_2 Biotrak Enzyme Immune Assay System (ELISA; Amersham Biosciences, Piscataway, NJ, USA). PGE_2 results were expressed as picograms per milligram of tissue wet weight (pg/mg tissue wet wt).

3.8. Nitric Oxide Synthase (NOS) Assay

NOS activity was measured in rat submandibular gland tissue by production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine according to the procedure described for brain slices [43]. Briefly, after 20-min preincubation in KRB solution, tissues were transferred to 500 mL of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of [U-^{14}C]-arginine (0.5 mCi). Drugs were added and the mixture incubated for 20 min under 5% CO_2 in O_2 at 37˚C. Tissues were then homogenized with an Ultraturrax homogenizer in 1 mL of medium containing 20 mM HEPES (pH 7.4), 0.5 mM ethyleneglycol tetra-acetic acid (EGTA), 0.5 mM ethylenediamine tetra-acetic acid (EDTA), 1 mM dithiothreitol, 1 mM leupeptin and 0.2 mM phenylmethylsulphonyl fluoride (PMSF) at 4˚C. After centrifugation at 20,000 × *g* for 10 min at 4˚C, supernatants were applied to 2-mL columns of Dowex AG 50 WX-8 (sodium form). [^{14}C]-citrulline was eluted with 3 mL of water and quantified by liquid scintillation counting. The results were expressed as picomol per gram tissue wet weight (pmol/g/tissue wet wt).

3.9. Statistical Analices

The unpaired Student's *t*-test was used to determine statistical significance. Analysis of variance (ANOVA) and a *post-hoc* test (Dunnett's method or the Student-Newman-Keuls test) were employed if a pairwise multiple comparison procedure was necessary. $p < 0.05$ was considered significant.

3.10. Ethical Approval of the Study Protocol

The study protocol was approved by the Ethics Committee of the School of Dentistry at Buenos Aires University (Buenos Aires, Argentina). The studies were conducted

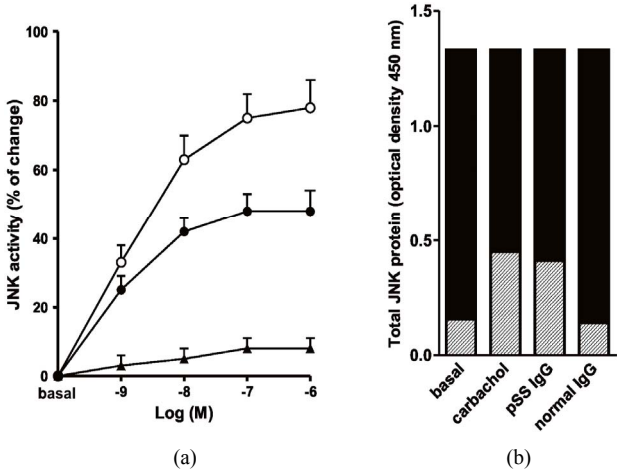
according to the tenets of the Declaration of Helsinki. All participants provided written informed consent to participate in the study.

4. Results

We initially determined the effects of different concentrations of carbachol and pSS IgG on JNK phosphorylation. **Figure 1** shows the potential of serum IgG from patients with pSS to stimulate JNK phosphorylation in a concentration-dependent manner. The authentic cholinergic agonist carbachol increased JNK activity (**Figure 1(a)**). The maximal effect of carbachol and/or pSS IgG on JNK activation was obtained at 1×10^{-6} M in both cases. The level of total JNK protein was not modified by pSS IgG, carbachol or normal IgG concentrations (**Figure 1(b)**). The data obtained with pSS IgG or carbachol therefore referred to their capacity to alter the level of JNK phosphorylation. The maximal capacity to stimulate the activity of the JNK enzyme in the presence of 1×10^{-6} M pSS IgG was impaired in the presence of 4.5×10^{-9} M of the specific M_3 mAChR antagonist J104192, but a lack of action was seen when the specific M_1 mAChR antagonist pirenzepine (1×10^{-6} M) was used (**Figure 2(a)**). Moreover, M_3 mAChR synthetic peptide (5×10^{-5} M) not M_1 synthetic peptide (5×10^{-5} M) blunted pSS IgG-inhibited JNK phosphorylation (**Figure 2(a)**).

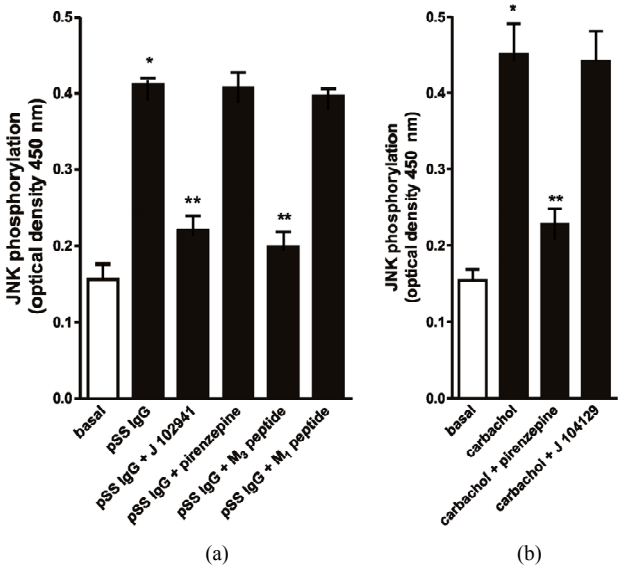
Carbachol (1×10^{-6} M)-stimulated JNK activity was blocked by the M_1 cholinergic antagonist pirenzepine (**Figure 2(b)**). To define the participation of NO, we studied which isoforms of NOS might be implicated in the action of pSS IgG on JNK enzyme activity. To achieve this, rat submandibular gland tissue was incubated with specific inhibitors of NOS isoforms. Inhibition of iNOS activity by methylisothiourea sulfate (1×10^{-7} M) prevented the stimulatory action of pSS IgG on JNK activation (**Figure 3(a)**). Conversely, inhibition of the activity of eNOS by L-NIO (5×10^{-6} M) and nNOS by NZ (5×10^{-5} M) was not observed. L-NMMA (1×10^{-5} M) prevented pSS IgG-mediated stimulation on JNK activity and a natural substrate of NOS, L-arginine (5×10^{-5} M), reversed the effect of L-NMMA (data no shown). pSS IgG induced an increase in the activity of NOS in submandibular gland tissue (**Figure 3(b)**). The stimulatory action of pSS IgG on NOS activity was abrogated by the inhibition of iNOS activity by methylisothiourea sulfate without modification by L-NIO and NZ. J104129 and M_3 synthetic peptide (but not pirenzepine and M_1 synthetic peptide) impaired the stimulatory action of pSS IgG. This indicated the participation of the M_3 mAChR in the action of pSS IgG upon NOS activity.

Figure 4(a) shows the participation of COX-2 (but not COX-1) in the stimulatory action of pSS IgG on JNK



(a) (b)

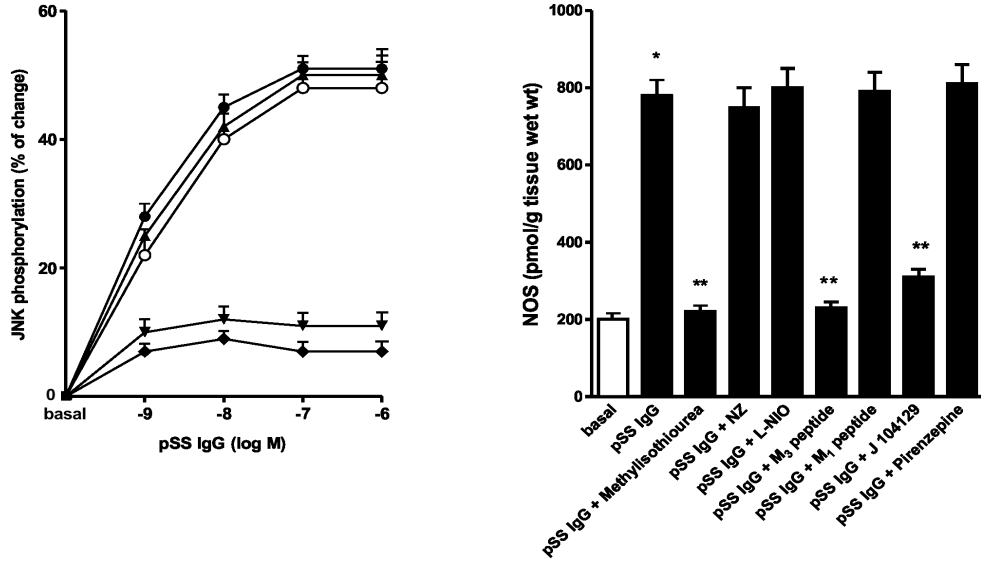
Figure 1. (a) Effect of pSS IgG (●), carbachol (○) and normal IgG (▲) on JNK activity in rat submandibular glands; (b) total JNK protein (black column) and JNK phosphorylated (striped line). Each point represents the mean ± SEM of six independent experiments done in duplicate.



(a) (b)

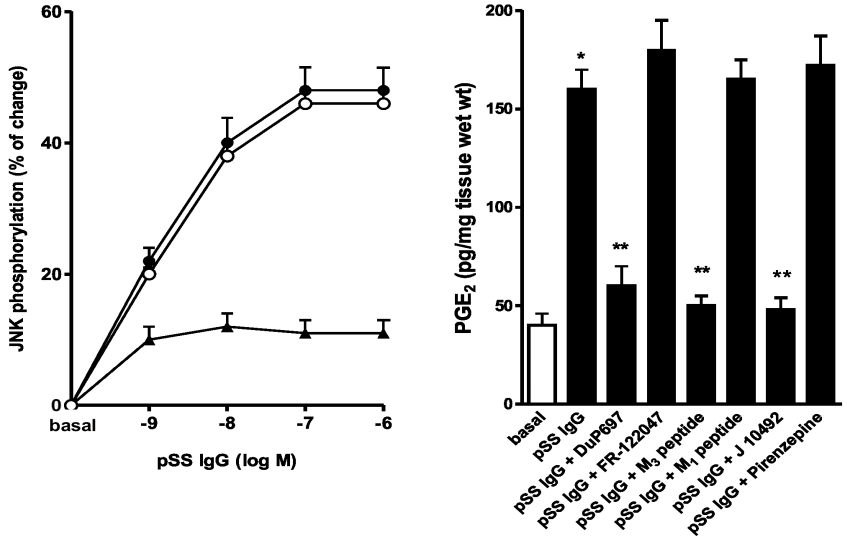
Figure 2. (a) Values of optical density of 1×10^{-6} M pSS IgG alone or in the presence of M_3 (J102941) and M_1 (pirenzepine) mAChR antagonists and M_3 and M_1 mAChR synthetic peptide; (b) optical density of 1×10^{-6} M carbachol alone or in the presence of M_3 (J102941) and M_1 (pirenzepine) mAChR antagonists. Results are mean ± SEM of 10 independent patients in each group done in duplicate. *$p < 0.001$ vs basal; **$p < 0.001$ vs pSS IgG or carbachol.

activity. A reduction in pSS IgG-mediated activation was observed in the presence of DuP 697 (5×10^{-8} M) but not by FR-122047 (5×10^{-6} M), which are COX-2 and COX-1 enzymatic inhibitors, respectively. pSS IgG (1×10^{-6} M) could trigger an increase in PGE_2 generation in rat submandibular glands, and COX-2 inhibition (but not COX-1



(a) (b)

Figure 3. (a) Concentration–response curves of pSS IgG alone (●) or in the presence of 1×10^{-4} M L-NMMA (♦), 1×10^{-7} M methylisothiourea sulfate (▼), 5×10^{-5} M NZ (○) and 5×10^{-6} M L-NIO (▲) on JNK phosphorylation; (b) stimulation of NOS activity by 1×10^{-6} M pSS IgG alone or in the presence of enzymatic inhibitors of NOS isoforms, M_3 and M_1 mAChR synthetic peptides, and M_3 and M_1 mAChR antagonists. Basal values A and B are also shown. Values represent the mean ± SEM of seven experiments in each group done in duplicate. *$p < 0.001$ vs basal; **$p < 0.0001$ vs pSS IgG.



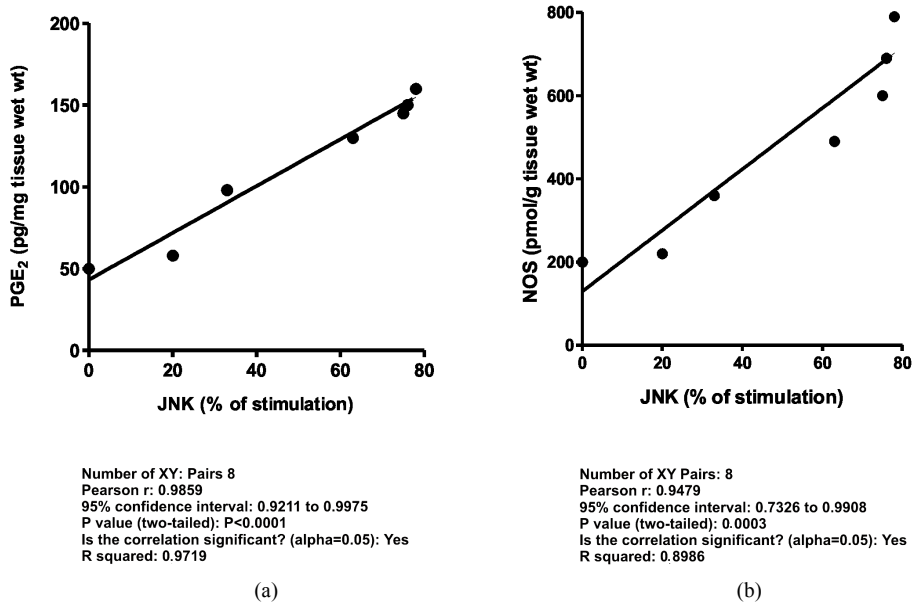
(a) (b)

Figure 4. (a) Concentration-response curves of pSS IgG alone (●) or in the presence of 5×10^{-8} M DuP 697 (▲) and 5×10^{-6} M FR-122047 (○) on JNK phosphorylation. (b) Stimulation of PGE_2 production by 1×10^{-6} M pSS IgG alone or in the presence of enzymatic inhibitors of COX-s isoforms, M_3 and M_1 mAChR synthetic peptides and M_3 and M_1 mAChR antagonists. Basal values ((a) and (b)) were also shown. Values represent the mean ± SEM of eight experiments in each group done in duplicate. *$p < 0.001$ vs basal; **$p < 0.0001$ vs pSS IgG.

inhibition) blunted this action of pSS IgG on PGE_2 production (**Figure 4(b)**). Also, the M_3 antagonist J104129 and M_3 synthetic peptide (but not the M_1 antagonist pirenzepine and M_1 synthetic peptide) diminished pSS IgG-stimulated PGE_2 production.

Figure 5 shows a positive correlation between the increment of NOS activity **Figure 5 (a)** and PGE_2 production **Figure 5(b)** in the function of JNK activation.

Table 1 shows the enzymatic pathways involved in the pSS IgG-mediated stimulation of JNK and carbachol-



(a) (b)

Figure 5. Correlation in the modulator effect of pSS IgG on JNK phosphorylation. Production of NOS and PGE_2 was plotted as a function of JNK activation. Values are the means of seven experiments in each group.

mediated stimulation of JNK. Inhibition of COX-1, NZ or L-NIO inhibited the stimulatory action of carbachol on JNK phosphorylation. M_3 and M_1 mAChR are calcium-mobilizing receptors coupled to PLC [44], so we determined the contribution of calcium to JNK phosphorylation by pSS IgG and carbachol in rat submandibular glands. Verapamil (1×10^{-5} M), an inhibitor of calcium influx and thapsigargin (1×10^{-8} M), an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase modified pSS IgG-mediated and carbachol-induced stimulation of JNK activity **(Table 2)**. On the other hand, calphostin C (5×10^{-9} M), an inhibitor of PKC, only modify pSS IgG-mediated activation of JNK (**Table 2**).

5. Discussion

Primary SS IgG with cholinergic agonist activity has been found in the sera of persons with Sjögren's syndrome (SS), and the presence of these antibodies correlated with inflammation of the salivary gland [6]. Here we demonstrated the possible role of pSS IgG to induce glandular inflammation through its capacity to trigger the production of the proinflammatory substances NO and PGE_2. Moreover, the increased production of these proinflammatory substances correlated with JNK phosphorylation.

Carbachol increased JNK activity mainly through the M_1 mAChR, but pSS IgG stimulated JNK activity predominantly through the M_3 mAChR. Inhibition of the M_3 mAChR by J104129 and M_1 mAChR by pirenzepine impaired pSS IgG and carbachol-induced increase in JNK phosphorylation.

Table 1. Enzymatic pathways coupled to the effect of pSS IgG and carbachol upon JNK phosphorylation.

Additions	JNK (% change)	
	pSS IgG (1×10^{-6} M)	Carbachol (1×10^{-6} M)
Alone	+163 ± 16	+ 189 ± 18
Methylisothiourea sulfate (1×10^{-7} M)	+70 ± 6.5*	+195 ± 17
Nz (5×10^{-5} M)	+161 ± 15	+140 ± 12*
L-NIO (5×10^{-6} M)	+167 ± 16	+130 ± 13*
DuP 697 (5×10^{-8} M)	+74 ± 6.8*	+182 ± 17
FR-122047 (5×10^{-6} M)	+159 ± 16	+136 ± 12*

Values are mean ± SEM of five experiments in each group carried out in duplicate. *$P < 0.001$ vs pSS IgG or carbachol alone.

Table 2. Contribution of calcium on the action of pSS IgG and carbachol upon JNK phosphorylation.

Additions	pSS IgG (1×10^{-6} M)	Carbachol (1×10^{-6} M)
Alone	+151 ± 15	186 ± 17
Verapamil (1×10^{-5} M)	+117 ± 12*	+124 ± 11*
Thapsigargin (1×10^{-6} M)	+132 ± 12	+122 ± 11**
Calphostin C (5×10^{-9} M)	+71 ± 8**	+182 ± 19

Values are mean ± SEM of six experiments in each group carried out in duplicate. *$P < 0.001$ vs alone. **$P < 0.0001$ vs alone.

The M_3 and M_1 mAChR subtypes are calcium-mobilizing receptors coupled to PLC activation [45]. They provide the second messenger inositol triphosphate (IP_3) and diacylglycerol (DAG) which mobilize intracellular calcium and activate PKC [41]. We observed that carbachol-phosphorylated JNK was diminished by calcium ATPase from a sarcoplasmic reticulum blocker whereas the pSS IgG-mediated effect was prevented by a PKC inhibitor. This indicated that JNK activation by M_1 mAChRs appeared to be dependent upon calcium/ calmodulin activity, whereas pSS IgG-activated JNK appeared to be involve M_3 mAChRs were associated with PKC activation. In support of our observations, it was shown that JNK activation in NIH3T3 cells by M_1 mAChRs did not require PKC [34]. However, PKC inhibition results in enhancement of JNK activity in CHO-M_3 cells [46,47].

In the present study, we showed that the activation of M_3 mAChRs in rat submandibular glands by pSS IgG increased generation of PGE_2. This was preceded by iNOS activation, which in turn catalyzed COX-2 activity. Our data indicated that iNOS dependent pathway was the key factor for pSS IgG-induced PGE_2 generation. Moreover, we demonstrated a positive correlation between NOS activity or PGE_2 production and the activation of JNK phosphorylation triggered by pSS IgG. The fact that inhibition of the activities of iNOS and COX-2 prevented the pSS IgG-mediated stimulation of JNK phosphorylation, parallel with an increase in the production of NO and PGE_2, confirmed this issue. Conversely, carbachol-stimulated JNK phosphorylation was inhibited by the activities of eNOS, nNOS and COX-1, indicating that the constitutive enzymes were involved.

eNOS and nNOS are constitutively expressed whereas the expression of iNOS requires protein synthesis. Furthermore, the reason why pSS IgG activated iNOS independent of an increase in intracellular calcium concentration was because this isoform could produce large amounts of NO for extended periods of time, far exceeding the levels generated by the constitutive isoforms in the submandibular gland [48]. Also, it has been documented that JNK regulates iNOS expression [49], it is likely that JNK mediates IL-1β-induced expression of iNOS in the lachrymal gland, which leads to inhibition of neural-as well as agonist-induced protein secretion [50].

The JNK cascade is triggered through pSS IgG and carbachol activating M_3 and M_1 mAChRs in rat submandibular glands. However, these stimuli, which trigger the production of NO and PGE_2, could activate the JNK cascade in the infiltrating T-cells in the salivary glands of pSS patients. The JNK cascade could play an important part in the pathogenesis of SS, and could be a potential therapeutic target [51,52].

6. Conclusions

We conclude that, in pSS, the early agonist-mediated activation of M_3 mAChRs initiated by autoantibodies binding to, and persistently activating, cholinoreceptors, resulted in JNK stimulation and an increase in the production of PGE_2 and NO through iNOS activation. This contributed to the inflammation of the submandibular gland, eliciting a loss of secretory response of glandular acini cells (dry mouth) and the lachrymal gland (dry eye) in patients with pSS. An illustration of bringing together the various systems studied and proposing a mechanism by which pSS IgG and carbachol might induce JNK activation, thereby triggering the production of proinflammatory mediators, is shown in **Figure 6**.

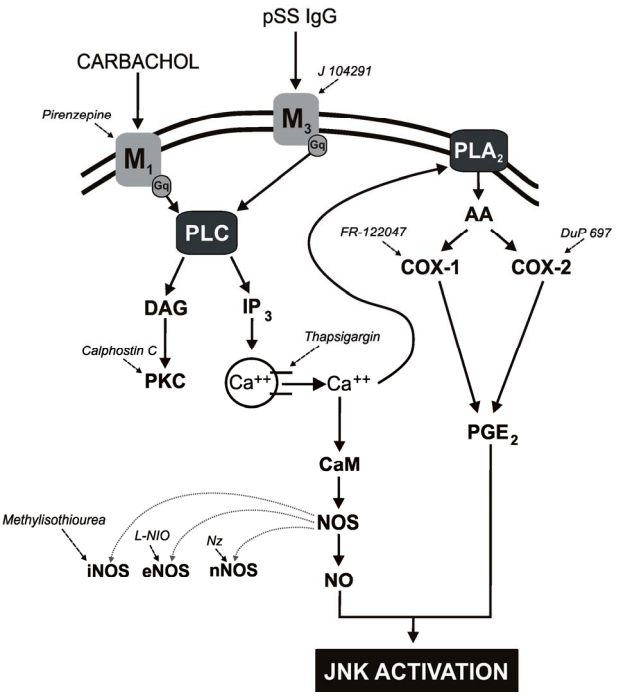


Figure 6. Proposed model to explain the mechanism whereby pSS IgG and carbachol up-regulates NOS isoform and PGE_2 generation to provoke JNK activation in submandibular gland. pSS IgG acting on M_3 mAChR and carbachol acting on M_1 mAChR activates PLC mediating production of 1,2-diacylglycerol (DAG) and inositol triphosphate (IP3). IP_3 triggering intracellular release of calcium stores (Ca^{2+}). Free calcium binds to calcium/calmodulin complex (CaM) and sensitizes PKC activation via DAG. Subsequent PKC translocation to the membrane and CaM complex increase NOS activity through different isoform that in turn increases NO production. The over production of NO also triggers COX-1 and COX-2 activation. Alternatively, the rise in cytosolic calcium activates phospholipase A_2 (PLA_2) with activation of COX-1 (carbachol) and COX-2 (pSS IgG) which induces generation of PGE_2, NO and PGE_2 in the last instance, evoked JNK activation. Inhibitory agents are indicated in italics.

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