

# Modulation of c-Jun NH<sub>2</sub>-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<sub>2</sub> 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<sub>2</sub> 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<sub>3</sub> mAChRs stimulated JNK phosphorylation whereas the activation of M<sub>1</sub> 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<sub>2</sub> 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<sub>2</sub>, 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<sub>1</sub> [3], M<sub>3</sub> [4,5] and M<sub>4</sub> [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 cholinergic receptors 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 cholinergic receptors initiated by autoantibodies [12] which bind to, and persistently activate, cholinergic receptors [13]. Subsequently, the agonistic activity displayed by these autoantibodies may induce the desensitization [14], internalization and/or intracellular degradation of cholinergic receptors, 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 lacrimal gland [16]. The infiltrating cells interfere with glandular function by cell-mediated glandular destruction and production of autoantibodies that interfere with cholinergic receptors [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<sub>1</sub>, M<sub>3</sub>, M<sub>5</sub>) through the activation of phospholipase C (PLC), which results in the release of the second messenger inositol 1,4,5-triphosphate (IP<sub>3</sub>) or by inhibition of adenylate cyclase (M<sub>2</sub>, M<sub>4</sub>), 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<sub>2</sub>-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)- $\alpha$ , 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<sub>1</sub> and M<sub>2</sub> mAChR [34-36], angiotensin [37],  $\alpha_1$ -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<sub>1</sub> and M<sub>3</sub> mAChRs were coupled to JNK in the submandibular glands of rats. However, carbachol preferentially stimulated M<sub>1</sub> mAChRs whereas pSS IgG stimulated M<sub>3</sub> mAChRs. Both, the activation of M<sub>3</sub> and M<sub>1</sub> 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<sub>3</sub> and M<sub>1</sub> 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 sialadenitis 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<sub>3</sub> mAChRs and M<sub>1</sub> 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) 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<sub>2</sub> in O<sub>2</sub> 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. PGE<sub>2</sub> Assay

Rat submandibular gland slices (20 mg) were incubated for 60 min in 500 µL of KRB and gassed with 5% CO<sub>2</sub> in O<sub>2</sub> 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<sub>2</sub> Biotrak Enzyme Immune Assay System (ELISA; Amersham Biosciences, Piscataway, NJ, USA). PGE<sub>2</sub> 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-<sup>14</sup>C]-citrulline from [U-<sup>14</sup>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 pre-warmed KRB equilibrated with 5% CO<sub>2</sub> in O<sub>2</sub> in the presence of [U-<sup>14</sup>C]-arginine (0.5 mCi). Drugs were added and the mixture incubated for 20 min under 5% CO<sub>2</sub> in O<sub>2</sub> 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). [<sup>14</sup>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 Analyses

The unpaired Student's *t*-test was used to determine statistical significance. Analysis of variance (ANOVA) and a *post-hoc* test (Dunnnett'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

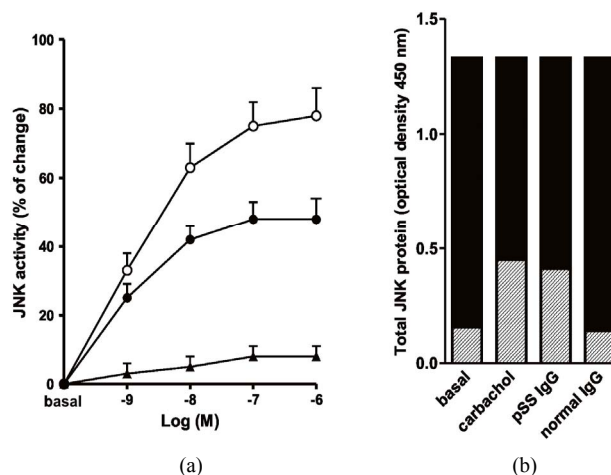
according to the tenets of the Declaration of Helsinki. All participants provided written informed consent to participate in the study.

#### 4. Results

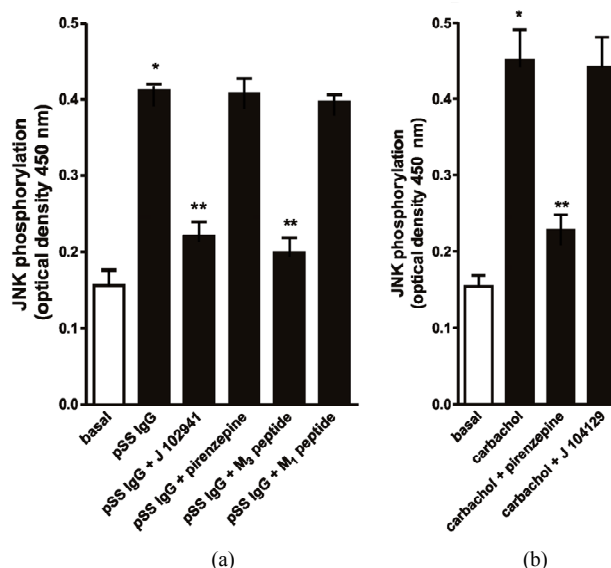
We initially determined the effects of different concentrations of carbachol and pSS IgG on JNK phosphorylation. **Figure 1** shows the potential of serum IgG from patients with pSS to stimulate JNK phosphorylation in a concentration-dependent manner. The authentic cholinergic agonist carbachol increased JNK activity (**Figure 1(a)**). The maximal effect of carbachol and/or pSS IgG on JNK activation was obtained at  $1 \times 10^{-6}$  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  $1 \times 10^{-6}$  M pSS IgG was impaired in the presence of  $4.5 \times 10^{-9}$  M of the specific M<sub>3</sub> mAChR antagonist J104192, but a lack of action was seen when the specific M<sub>1</sub> mAChR antagonist pirenzepine ( $1 \times 10^{-6}$  M) was used (**Figure 2(a)**). Moreover, M<sub>3</sub> mAChR synthetic peptide ( $5 \times 10^{-5}$  M) not M<sub>1</sub> synthetic peptide ( $5 \times 10^{-5}$  M) blunted pSS IgG-inhibited JNK phosphorylation (**Figure 2(a)**).

Carbachol ( $1 \times 10^{-6}$  M)-stimulated JNK activity was blocked by the M<sub>1</sub> 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 ( $1 \times 10^{-7}$  M) prevented the stimulatory action of pSS IgG on JNK activation (**Figure 3(a)**). Conversely, inhibition of the activity of eNOS by L-NIO ( $5 \times 10^{-6}$  M) and nNOS by NZ ( $5 \times 10^{-5}$  M) was not observed. L-NMMA ( $1 \times 10^{-5}$  M) prevented pSS IgG-mediated stimulation on JNK activity and a natural substrate of NOS, L-arginine ( $5 \times 10^{-5}$  M), reversed the effect of L-NMMA (data not 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<sub>3</sub> synthetic peptide (but not pirenzepine and M<sub>1</sub> synthetic peptide) impaired the stimulatory action of pSS IgG. This indicated the participation of the M<sub>3</sub> 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

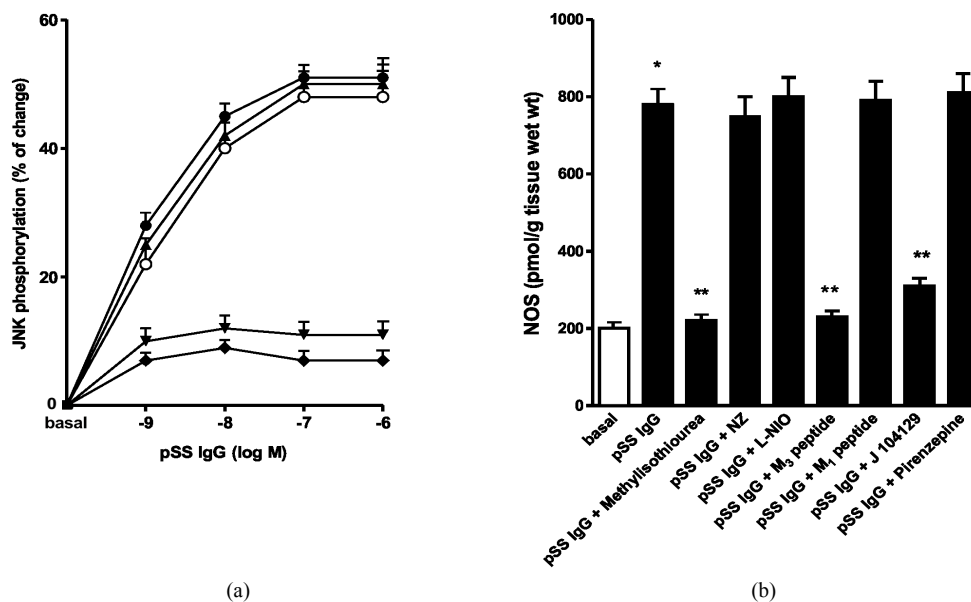


**Figure 1.** (a) Effect of pSS IgG (●), carbachol (○) and normal IgG (▲) on JNK activity in rat submandibular glands; (b) total JNK protein (black column) and JNK phosphorylated (striped line). Each point represents the mean  $\pm$  SEM of six independent experiments done in duplicate.

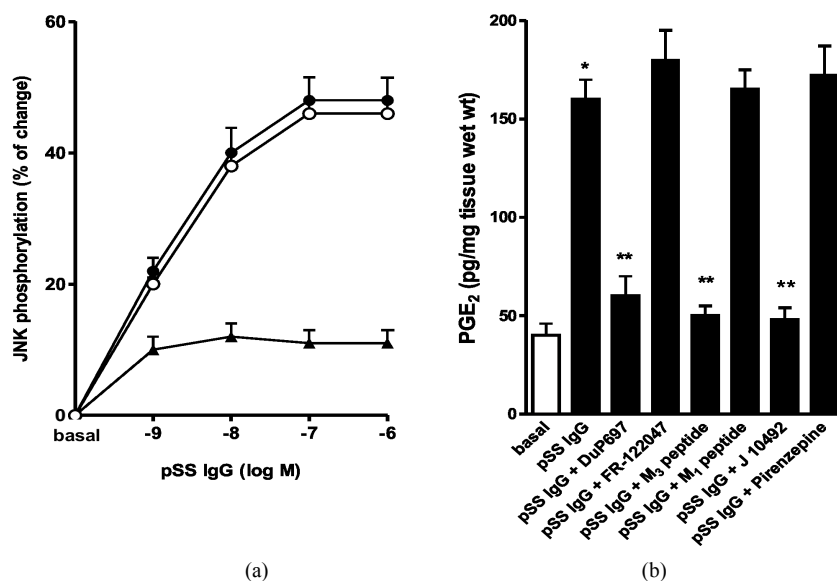


**Figure 2.** (a) Values of optical density of  $1 \times 10^{-6}$  M pSS IgG alone or in the presence of M<sub>3</sub> (J102941) and M<sub>1</sub> (pirenzepine) mAChR antagonists and M<sub>3</sub> and M<sub>1</sub> mAChR synthetic peptide; (b) optical density of  $1 \times 10^{-6}$  M carbachol alone or in the presence of M<sub>3</sub> (J102941) and M<sub>1</sub> (pirenzepine) mAChR antagonists. Results are mean  $\pm$  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 ( $5 \times 10^{-8}$  M) but not by FR-122047 ( $5 \times 10^{-6}$  M), which are COX-2 and COX-1 enzymatic inhibitors, respectively. pSS IgG ( $1 \times 10^{-6}$  M) could trigger an increase in PGE<sub>2</sub> generation in rat submandibular glands, and COX-2 inhibition (but not COX-1



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

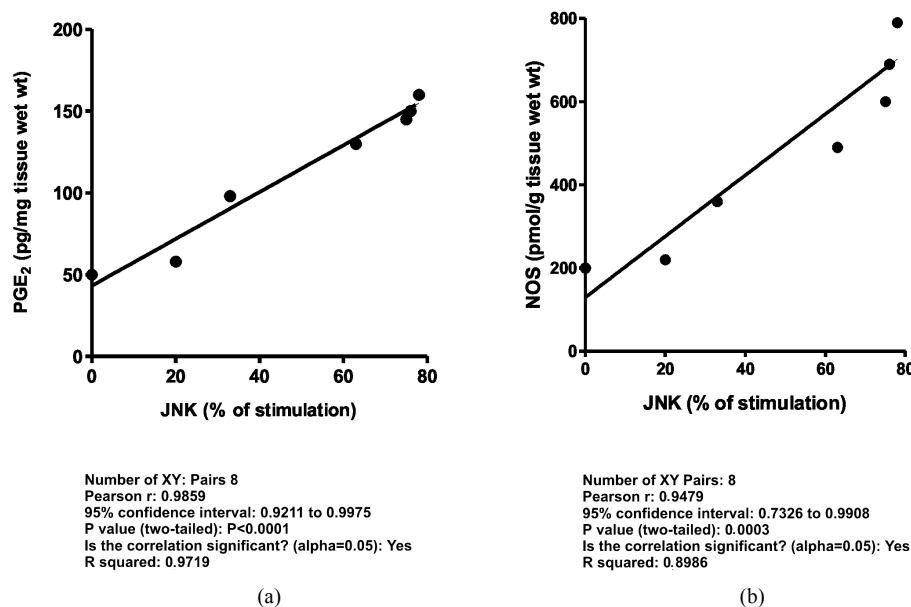


**Figure 4.** (a) Concentration-response curves of pSS IgG alone (●) or in the presence of  $5 \times 10^{-8}$  M DuP 697 (▲) and  $5 \times 10^{-6}$  M FR-122047 (○) on JNK phosphorylation. (b) Stimulation of PGE<sub>2</sub> production by  $1 \times 10^{-6}$  M pSS IgG alone or in the presence of enzymatic inhibitors of COX-s isoforms, M<sub>3</sub> and M<sub>1</sub> mAChR synthetic peptides and M<sub>3</sub> and M<sub>1</sub> mAChR antagonists. Basal values ((a) and (b)) were also shown. Values represent the mean  $\pm$  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<sub>2</sub> production (Figure 4(b)). Also, the M<sub>3</sub> antagonist J104129 and M<sub>3</sub> synthetic peptide (but not the M<sub>1</sub> antagonist pirenzepine and M<sub>1</sub> synthetic peptide) diminished pSS IgG-stimulated PGE<sub>2</sub> production.

Figure 5 shows a positive correlation between the increment of NOS activity Figure 5 (a) and PGE<sub>2</sub> 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-



**Figure 5.** Correlation in the modulator effect of pSS IgG on JNK phosphorylation. Production of NOS and PGE<sub>2</sub> 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<sub>3</sub> and M<sub>1</sub> 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 ( $1 \times 10^{-5}$  M), an inhibitor of calcium influx and thapsigargin ( $1 \times 10^{-8}$  M), an inhibitor of endoplasmic reticulum Ca<sup>2+</sup>-ATPase modified pSS IgG-mediated and carbachol-induced stimulation of JNK activity (**Table 2**). On the other hand, calphostin C ( $5 \times 10^{-9}$  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<sub>2</sub>. Moreover, the increased production of these proinflammatory substances correlated with JNK phosphorylation.

Carbachol increased JNK activity mainly through the M<sub>1</sub> mAChR, but pSS IgG stimulated JNK activity predominantly through the M<sub>3</sub> mAChR. Inhibition of the M<sub>3</sub> mAChR by J104129 and M<sub>1</sub> 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 ( $1 \times 10^{-6}$ M)	Carbachol ( $1 \times 10^{-6}$ M)
Alone	+163 ± 16	+189 ± 18
Methylisothiourea sulfate ( $1 \times 10^{-7}$ M)	+70 ± 6.5*	+195 ± 17
Nz ( $5 \times 10^{-5}$ M)	+161 ± 15	+140 ± 12*
L-NIO ( $5 \times 10^{-6}$ M)	+167 ± 16	+130 ± 13*
DuP 697 ( $5 \times 10^{-8}$ M)	+74 ± 6.8*	+182 ± 17
FR-122047 ( $5 \times 10^{-6}$ 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	JNK (% change)	
	pSS IgG ( $1 \times 10^{-6}$ M)	Carbachol ( $1 \times 10^{-6}$ M)
Alone	+151 ± 15	186 ± 17
Verapamil ( $1 \times 10^{-5}$ M)	+117 ± 12*	+124 ± 11*
Thapsigargin ( $1 \times 10^{-6}$ M)	+132 ± 12	+122 ± 11**
Calphostin C ( $5 \times 10^{-9}$ 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<sub>3</sub> and M<sub>1</sub> mAChR subtypes are calcium-mobilizing receptors coupled to PLC activation [45]. They provide the second messenger inositol triphosphate (IP<sub>3</sub>) 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<sub>1</sub> mAChRs appeared to be dependent upon calcium/calmodulin activity, whereas pSS IgG-activated JNK appeared to be involve M<sub>3</sub> mAChRs were associated with PKC activation. In support of our observations, it was shown that JNK activation in NIH3T3 cells by M<sub>1</sub> mAChRs did not require PKC [34]. However, PKC inhibition results in enhancement of JNK activity in CHO-M<sub>3</sub> cells [46,47].

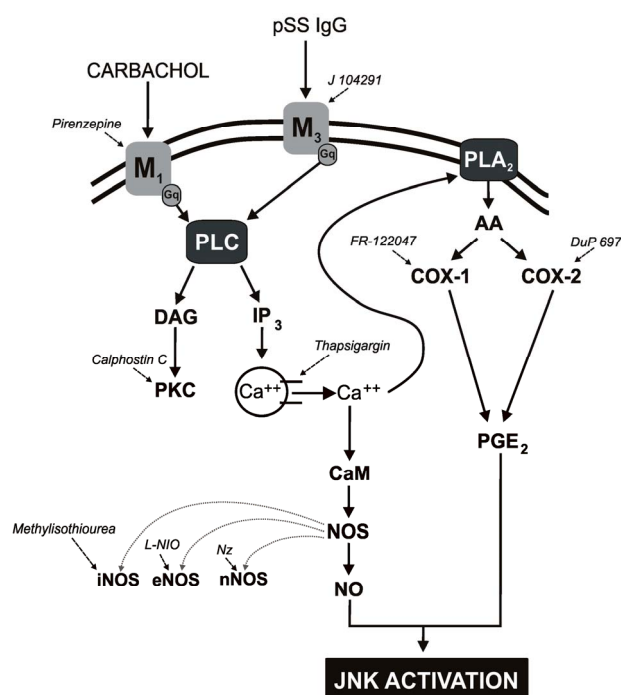
In the present study, we showed that the activation of M<sub>3</sub> mAChRs in rat submandibular glands by pSS IgG increased generation of PGE<sub>2</sub>. This was preceded by iNOS activation, which in turn catalyzed COX-2 activity. Our data indicated that iNOS dependent pathway was the key factor for pSS IgG-induced PGE<sub>2</sub> generation. Moreover, we demonstrated a positive correlation between NOS activity or PGE<sub>2</sub> 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<sub>2</sub>, 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-1 $\beta$ -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<sub>3</sub> and M<sub>1</sub> mAChRs in rat submandibular glands. However, these stimuli, which trigger the production of NO and PGE<sub>2</sub>, 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<sub>3</sub> mAChRs initiated by autoantibodies binding to, and persistently activating, cholinergic receptors, resulted in JNK stimulation and an increase in the production of PGE<sub>2</sub> 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<sub>2</sub> generation to provoke JNK activation in submandibular gland. pSS IgG acting on M<sub>3</sub> mAChR and carbachol acting on M<sub>1</sub> mAChR activates PLC mediating production of 1,2-diacylglycerol (DAG) and inositol triphosphate (IP<sub>3</sub>). IP<sub>3</sub> triggering intracellular release of calcium stores (Ca<sup>2+</sup>). 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<sub>2</sub> (PLA<sub>2</sub>) with activation of COX-1 (carbachol) and COX-2 (pSS IgG) which induces generation of PGE<sub>2</sub>, NO and PGE<sub>2</sub> in the last instance, evoked JNK activation. Inhibitory agents are indicated in italics.

## 7. Acknowledgements

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## REFERENCES

- [1] I. Marczinovits, L. Kovacs, A. Gyorgy, G. K. Toth, L. Dorgai and J. A. Molnar, "A Peptide of Human Muscarinic Acetylcholine Receptor 3 Is Antigenic in Primary Sjögren's Syndrome," *Journal of Autoimmunity*, Vol. 24, No. 1, 2005, pp. 47-54. [doi:10.1016/j.jaut.2004.11.005](https://doi.org/10.1016/j.jaut.2004.11.005)
- [2] H. M. Moutsopoulos and N. Talal, "Immunologic Abnormalities in Sjögren Syndrome," In: N. Talal and H. M. Moutsopoulos, Eds., *Sjögren's Syndrome: Clinical and immunopathological Aspects*, Springer-Verlag, Berlin, 1987, pp. 258-265.
- [3] C. P. Leiros, L. Sterin-Borda, E. S. Borda, J. C. Goin and M. M. Hosey, "Desensitization and Sequestration of Human M<sub>2</sub> Muscarinic Acetylcholine Receptors by Autoantibodies from Patients with Chagas' Disease," *Journal of Biology and Chemistry*, Vol. 272, No. 20, 1999, pp. 12989-12993.
- [4] S. Bacman, L. Sterin-Borda, J. J. Camusso, R. Arana, O. Hubscher and E. Borda, "Circulating Antibodies against Rat Parotid Gland M<sub>3</sub> Muscarinic Receptors in Sjögren Syndrome," *Clinical & Experimental Immunology*, Vol. 104, No. 3, 1996, pp. 454-459. [doi:10.1046/j.1365-2249.1996.42748.x](https://doi.org/10.1046/j.1365-2249.1996.42748.x)
- [5] S. Bacman, A. Berra, L. Sterin-Borda and E. Borda, "Muscarinic Acetylcholine Receptor Antibodies as a New Marker of Dry Eye Sjögren Syndrome," *Investigative Ophthalmology & Vision Science*, Vol. 42, No. 2, 2001, pp. 321-327.
- [6] S. Reina, L. Sterin-Borda, B. Orman and E. Borda, "Autoantibodies against Submandibular Gland Muscarinic Cholinergic Receptor Subtypes in Primary Sjögren," *European Journal of Inflammation*, Vol. 3, No. 3, 2005, pp. 135-141.
- [7] L. Kovacs, I. Marczinovits, A. Gryorgy, G. Toth, L. Dorgai and J. Pal, "Clinical Associations of Autoantibodies to Human Muscarinic Acetylcholine Receptor M<sub>3</sub> in Primary Sjögren's Syndrome," *Rheumatology*, Vol. 44, No. 8, 2005, pp. 1021-1025. [doi:10.1093/rheumatology/keh672](https://doi.org/10.1093/rheumatology/keh672)
- [8] S. Reina, L. Sterin-Borda, B. Orman and E. Borda, "Human mAChR Antibodies from Sjögren Syndrome Sera Increase Cerebral Nitric Oxide Synthase Activity and Nitric Oxide Synthase mRNA Level," *Clinical Immunology*, Vol. 113, No. 2, 2004, pp. 193-202. [doi:10.1016/j.clim.2004.08.005](https://doi.org/10.1016/j.clim.2004.08.005)
- [9] S. Reina, B. Orman, L. Anaya, L. Sterin-Borda and E. Borda, "Cholinergic Autoantibodies in Sjögren Syndrome," *Journal of Dental Research*, Vol. 86, No. 9, 2007, pp. 832-836. [doi:10.1177/154405910708600905](https://doi.org/10.1177/154405910708600905)
- [10] A. Berra, L. Sterin-Borda, S. Bacman and E. Borda, "Role of Salivary IgA in the Pathogenesis of Sjögren Syndrome," *Clinical Immunology*, Vol. 104, No. 1, 2002, pp. 49-57. [doi:10.1006/clim.2002.5228](https://doi.org/10.1006/clim.2002.5228)
- [11] L. J. Dawson, J. Stanbury, N. Venn, B. Hasdimir, S. N. Rogers and P. M. Smith, "Antimuscarinic Antibodies in Primary Sjögren's Syndrome Reversibly Inhibit the Mechanism of Fluid Secretion by Human Submandibular Salivary Acinar Cells," *Arthritis & Rheumatology*, Vol. 54, No. 4, 2006, pp. 1165-1173. [doi:10.1002/art.21764](https://doi.org/10.1002/art.21764)
- [12] J. Li, Y. M. Ha, N. Y. Ku, S. Y. Choi, S. J. Lee, S. B. Oh, et al. "Inhibitory Effects of Autoantibodies on the Muscarinic Receptors in Sjögren's Syndrome," *Laboratory Investigation*, Vol. 84, No. 11, 2004, pp. 1430-1438. [doi:10.1038/labinvest.3700173](https://doi.org/10.1038/labinvest.3700173)
- [13] S. A. Waterman, T. P. Gordon and M. Rischmueller, "Inhibitory Effects of Muscarinic Receptor Autoantibodies on Parasympathetic Neurotransmission in Sjögren's Syndrome," *Arthritis & Rheumatology*, Vol. 43, No. 7, 2000, pp. 1647-1654. [doi:10.1002/1529-0131\(200007\)43:7<1647::AID-ANR31>3.0.CO;2-P](https://doi.org/10.1002/1529-0131(200007)43:7<1647::AID-ANR31>3.0.CO;2-P)
- [14] S. Cha, E. Singson, J. Cornelius, J. P. Yagna, H. J. Knot and A. B. Peck, "Muscarinic Acetylcholine Type-3 Receptor Desensitization Due to Chronic Exposure to Sjögren Syndrome-Associated Autoantibodies," *The Journal of Rheumatology*, Vol. 33, No. 2, 2006, pp. 296-306.
- [15] D. B. Ferguson, "The Flow Rate and Composition of Human Labial Gland Saliva," *Archives of Oral Biology*, Vol. 44, Supplement 1, 1999, pp. S11-S14. [doi:10.1016/S0003-9969\(99\)90004-3](https://doi.org/10.1016/S0003-9969(99)90004-3)
- [16] K. Tsubota, M. Kaido, Y. Yagi and T. Fujihara, "Diseases Associated with Ocular Surface Abnormalities: The Importance of Reflex Tearing," *British Journal of Ophthalmology*, Vol. 83, No. 1, 1999, pp. 89-91. [doi:10.1136/bjo.83.1.89](https://doi.org/10.1136/bjo.83.1.89)
- [17] R. I. Fox, "Sjögren's Syndrome," *The Lancet*, Vol. 366, No. 9482, 2005, pp. 321-331. [doi:10.1016/S0140-6736\(05\)66990-5](https://doi.org/10.1016/S0140-6736(05)66990-5)
- [18] N. Ravald and T. List, "Caries and Periodontal Conditions in Patients with Primary Sjögren's Syndrome," *Sweden Dental Journal*, Vol. 22, No. 3, 1998, pp. 97-103.
- [19] J. Yuan, A. S. Bowman, M. Aljamali, M. R. Payne, J. S. Tucker, J. W. Dillwith, et al. "Prostaglandin E(2)-Stimulated Secretion of Protein in Salivary Glands of the Lone Star Tick via a Phosphoinositide Signaling Pathway," *Insect Biochemical and Molecular Biology*, Vol. 30, No. 11, 2000, pp. 1099-1106.
- [20] G. M. Pasinetti, "Cyclooxygenase and Alzheimer's Disease: Implications for Preventive Initiatives to Slow the Progression of Clinical Dementia," *Archives of Gerontology and Geriatric*, Vol. 33, No. 1, 2001, pp. 13-28. [doi:10.1016/S0167-4943\(01\)00091-7](https://doi.org/10.1016/S0167-4943(01)00091-7)
- [21] V. Mollage, C. E. Muscoli, E. Masini, S. Cuzzocrea and D. Salvemini, "Modulation of Prostaglandin Biosynthesis by Nitric Oxide and Nitric Oxide Donors," *Pharmacological Review*, Vol. 57, No. 2, 2005, pp. 217-252.



- [doi:10.1124/pr.57.2.1](https://doi.org/10.1124/pr.57.2.1)
- [22] S. Ganzinelli, E. Borda and L. Sterin-Borda, "Autoantibodies from Schizophrenia Patients Induce Cerebral Cox-1/iNOS mRNA Expression with NO/PGE<sub>2</sub>/MMP-3 Production," *The International Journal of Neuropsychopharmacology*, Vol. 13, No. 3, 2010, pp. 293-303. [doi:10.1017/S1461145709990770](https://doi.org/10.1017/S1461145709990770)
- [23] E. C. Hulme, N. J. Birdsall and N. J. Buckley, "Muscarinic Receptor Subtypes," *Annual Review of Pharmacology and Toxicology*, Vol. 30, No. 1, 1990, pp. 633-673. [doi:10.1146/annurev.pa.30.040190.003221](https://doi.org/10.1146/annurev.pa.30.040190.003221)
- [24] B. Nicke, K. Detjen and C. D. Logsdon, "Muscarinic Cholinergic Receptors Activate Both Inhibitory and stimulatory Growth Mechanisms in NIH<sub>3</sub>T<sub>3</sub> Cells," *The Journal of Biology and Chemistry*, Vol. 274, No. 31, 1999, pp. 21701-21706. [doi:10.1074/jbc.274.31.21701](https://doi.org/10.1074/jbc.274.31.21701)
- [25] J. S. Gutkind and K. C. Robbins, "Activation of Transforming G Protein-Coupled Receptors Induces Rapid Tyrosine Phosphorylation of Cellular Proteins, Including p125FAK and the p130 v-src Substrate," *Biochemistry and Biophysical Research Communication*, Vol. 188, No. 1, 1992, pp.155-161. [doi:10.1016/0006-291X\(92\)92363-3](https://doi.org/10.1016/0006-291X(92)92363-3)
- [26] R. J. Davis, "The Mitogen-Activated Protein Kinase Signal Transduction Pathway," *The Journal of Biology and Chemistry*, Vol. 268, No. 20, 1993, pp. 14553-14556.
- [27] A. Minden and M. Karin, "Regulation and Function of the JNK Subgroup of MAP Kinases," *Biochimica et Biophysica Acta*, Vol. 1333, No. 2, 1993, pp. F85-F104.
- [28] S. Gupta, T. Barrett, A. J. Whitmarsh, J. Cavanagh, H. K. Sluss, B. Dérjard and R. J. Davis, "Selective Interaction of JNK Protein Kinase Isoforms with Transcription Factors," *The EMBO Journal*, Vol. 15, No. 11, 1996, pp. 2760-2770.
- [29] J. M. Kyriakis, P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch and J. R. Woodgett. "The Stress-Activated Protein Kinase Subfamily of c-Jun Kinases," *Nature*, Vol. 369, No. 6476, 1994, pp. 156-160. [doi:10.1038/369156a0](https://doi.org/10.1038/369156a0)
- [30] J. M. Kyriakis and J. Avruch, "Sounding the Alarm: Protein Kinase Cascades Activated by Stress and Inflammation," *Journal of Biology and Chemistry*, Vol. 271, No. 40, 1996, pp. 24313-24316. [doi:10.1074/jbc.271.40.24313](https://doi.org/10.1074/jbc.271.40.24313)
- [31] A. Paul, S. Wilson, C. M. Belham, C. J. Robinson, P. H. Scott, G. W. Gould and R. Plevin, "Stress-Activated Protein Kinases: Activation, Regulation and Function," *Cellular Signalling*, Vol. 9, No. 6, 1997, pp. 403-410. [doi:10.1016/S0898-6568\(97\)00042-9](https://doi.org/10.1016/S0898-6568(97)00042-9)
- [32] C. A. Dinarello, "Proinflammatory Cytokines," *Chest*, Vol. 118, No. 2, 2000, pp. 503-508. [doi:10.1378/chest.118.2.503](https://doi.org/10.1378/chest.118.2.503)
- [33] J. S. Gutkind, "The Pathways Connecting G Protein-Coupled Receptors to the Nucleus through Divergent Mitogen-Activated Protein Kinase Cascades," *The Journal of Biology and Chemistry*, Vol. 273, No. 4, 1998, pp. 1839-1842. [doi:10.1074/jbc.273.4.1839](https://doi.org/10.1074/jbc.273.4.1839)
- [34] O. A. Coso, M. Chiariello, G. Kalinec, J. M. Kyriakis, J. Woodgett and J. S. Gutkind. "Transforming G Protein-Coupled Receptors Potently Activate JNK (SAPK). Evidence for a Divergence from the Tyrosine Kinase Signaling Pathway," *The Journal of Biology and Chemistry*, Vol. 270, No. 10, 1995, pp. 5620-5624.
- [35] F. M. Mitchell, M. Russell and G. L. Johnson, "Differential Calcium Dependence in the Activation of c-Jun Kinase and Mitogen-Activated Protein Kinase by Muscarinic Acetylcholine Receptors in Rat 1A Cells," *Biochemistry Journal*, Vol. 309, Part 2, 1995, pp. 381-384.
- [36] O. A. Coso, H. Teramoto, W. F. Simonds and J. S. Gutkind, "Signaling from G Protein-Coupled Receptors to c-Jun Kinase Involves Beta Gamma Subunits of Heterotrimeric G Proteins Acting on a Ras and Rac1-dependent Pathway," *The Journal of Biology and Chemistry*, Vol. 271, No. 8, 1996, pp. 3963-3966. [doi:10.1074/jbc.271.8.3963](https://doi.org/10.1074/jbc.271.8.3963)
- [37] I. E. Zohn, H. Yu, X. Li, A. D. Cox and H. S. Earp, "Angiotensin II Stimulates Calcium-Dependent Activation of c-Jun N-Terminal Kinase," *Molecular and Cellular Biology*, Vol. 15, No. 11, 1995, pp. 6160-6168.
- [38] M. T. Ramirez, V. P. Sah, X. L. Zhao, J. J. Hunter, K. R. Chien and J. H. Brown, "The MEKK-JNK Pathway is Stimulated by Alpha1-Adrenergic Receptor and Ras Activation and Is Associated with *in Vitro* and *in Vivo* Cardiac Hypertrophy," *The Journal of Biology and Chemistry*, Vol. 272, No. 22, 1997, pp. 14057-14061. [doi:10.1074/jbc.272.22.14057](https://doi.org/10.1074/jbc.272.22.14057)
- [39] P. S. Shapiro, J. N. Evans, R. J. Davis and J. A. Posada, "The Seven-Transmembrane-Spanning Receptors for Endothelin and Thrombin Cause Proliferation of Airway Smooth Muscle Cells and Activation of the Extracellular Regulated Kinase and c-Jun NH<sub>2</sub>-Terminal Kinase Groups of mitogen-Activated Protein Kinases," *The Journal of Biology and Chemistry*, Vol. 271, No. 10, 1996, pp. 5750-5754. [doi:10.1074/jbc.271.10.5750](https://doi.org/10.1074/jbc.271.10.5750)
- [40] M. A. Bogoyevitch, A. J. Ketterman and P. H. Sugden, "Cellular Stresses Differentially Activate c-Jun N-Terminal Protein Kinases and Extracellular Signal-Regulated Protein Kinases in Cultured Ventricular Myocytes," *The Journal of Biology and Chemistry*, Vol. 270, No. 50, 1995, pp. 29710-29717. [doi:10.1074/jbc.270.50.29710](https://doi.org/10.1074/jbc.270.50.29710)
- [41] A. C. Newton, "Protein Kinase C: Structure, Function, and Regulation," *The Journal of Biology and Chemistry*, Vol. 270, No. 48, 1995, pp. 28495-28498.
- [42] C. Vitali, S. Bombardieri, H. M. Moutsopoulos, G. Balestrieri, W. Bencivelli, R. M. Bernstein, *et al.* "Preliminary Criteria for the Classification of Sjögren's Syndrome. Results from a Prospective Concerted Action Supported by the European Community," *Arthritis and Rheumatology*, Vol. 36, No. 3, 1993, pp. 340-347. [doi:10.1002/art.1780360309](https://doi.org/10.1002/art.1780360309)
- [43] T. Borda, A. Genaro, L. Sterin-Borda and G. Cremaschi, "Involvement of Endogenous Nitric Oxide Signalling System in Brain Muscarinic Acetylcholine Receptor Activation," *Journal of Neural Transmission*, Vol. 105, No. 2-3, 1995, pp. 193-204. [doi:10.1007/s007020050048](https://doi.org/10.1007/s007020050048)
- [44] M. P. Caulfield, "Muscarinic Receptor Characterization,

- Coupling and Function," *Pharmacology & Therapeutic*, Vol. 58, No. 3, 1993, pp. 319-379.  
[doi:10.1016/0163-7258\(93\)90027-B](https://doi.org/10.1016/0163-7258(93)90027-B)
- [45] M. J. Berridge, "Inositol Trisphosphate and Calcium Signalling," *Nature*, Vol. 361, No. 6410, 1993, pp. 315-325.  
[doi:10.1038/361315a0](https://doi.org/10.1038/361315a0)
- [46] P. G. Wylie, R. A. Challiss and J. L. Blank, "Regulation of Extracellular-Signal Regulated Kinase and c-Jun N-Terminal Kinase by G-Protein-Linked Muscarinic Acetylcholine Receptors," *Biochemistry Journal*, Vol. 338, Part 3, 1999, pp. 619-628. [doi:10.1042/0264-6021:3380619](https://doi.org/10.1042/0264-6021:3380619)
- [47] D. C. Hornigold, R. Mistry, P. D. Raymond, J. L. Blank and R. A. Challiss, "Evidence for Cross-Talk between M<sub>2</sub> and M<sub>3</sub> Muscarinic Acetylcholine Receptors in the Regulation of Second Messenger and Extracellular Signal-Regulated Kinase Signalling Pathways in Chinese Hamster Ovary Cells," *British Journal of Pharmacology*, Vol. 138, No. 7, 2003, pp. 1340-1350.  
[doi:10.1038/sj.bjp.0705178](https://doi.org/10.1038/sj.bjp.0705178)
- [48] P. Kubes and D. M. McCafferty, "Nitric Oxide and Intestinal Inflammation," *The American Journal of Medicine*, Vol. 109, No. 2, 2000, pp. 150-118.  
[doi:10.1016/S0002-9343\(00\)00480-0](https://doi.org/10.1016/S0002-9343(00)00480-0)
- [49] M. T. Heneka and D. L. Feinstein, "Expression and Function of Inducible Nitric Oxide Synthase in Neurons," *Journal of Neuroimmunology*, Vol. 114, No. 1, 2001, pp. 8-18. [doi:10.1016/S0165-5728\(01\)00246-6](https://doi.org/10.1016/S0165-5728(01)00246-6)
- [50] D. Zoukhri, E. Macari, S. H. Choi and C. L. Kublin, "c-Jun NH<sub>2</sub>-Terminal Kinase Mediates Interleukin-1beta-Induced Inhibition of Lacrimal Gland Secretion," *Journal of Neurochemistry*, Vol. 96, No. 1, 2006, pp. 126-135.  
[doi:10.1111/j.1471-4159.2005.03529.x](https://doi.org/10.1111/j.1471-4159.2005.03529.x)
- [51] K. Soejima, H. Nakamura, M. Tamai, A. Kawakami and K. Eguchi, "Activation of MKK4 (SEK1), JNK, and c-Jun in Labial Salivary Infiltrating T Cells in Patients with Sjögren's Syndrome," *Rheumatology International*, Vol. 27, No. 4, 2006, pp. 329-333.
- [52] S. Gonzalo, L. Grasa, M. P. Arrueba, M. A. Plaza and M. D. Murillo, "Lipopolysaccharide-Induced Digestive and Liver Disease," *Digestive and Liver Disease*, Vol. 43, No. 4, 2011, pp. 277-285. [doi:10.1016/j.dld.2010.10.009](https://doi.org/10.1016/j.dld.2010.10.009)