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International Immunopharmacology 7 (2007) 1535–1543

International Immunopharmacology

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Anti-brain cholinergic auto antibodies from primary Sjögren syndrome sera modify simultaneously cerebral nitric oxide and prostaglandin biosynthesis

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Received 30 May 2007; received in revised form 18 July 2007; accepted 23 July 2007

Abstract

The presence of circulating antibodies from primary Sjögren Syndrome (pSS) patients enable to interact with rat cerebral frontal cortex by activating muscarinic acetylcholine receptors (mAChR). ELISA assay for PGE_2 generation, nitric oxide synthase (NOS) activity was measured in cerebral frontal cortex slices by production of [U−^{14}C]-citruline and mRNA isolation/quantitative PCR for COX-1 and COX-2 gene expression were carried out. By ELISA assay, it was shown that IgG from pSS patients reacted to cerebral frontal cortex cell surface and with human M_1 and M_3 mAChR. Beside pSS IgG displayed an agonistic-like activity stimulating NOS activity and PGE_2 production associated with an increased COX-1 mRNA gene expression, without affecting COX-2 mRNA levels. Inhibition of phospholipase A_2 (PLA_2) and NOS prevented pSS IgG effects upon both PGE_2 production and COX-1 mRNA levels. The results support the notion that serum IgG auto antibodies in pSS patients target cerebral mAChR may have pathogenic role in immune neuroinflammation and on cognitive dysfunction present in pSS patients.

Keywords: Antibodies; Sjögren syndrome; mAChR; Prostaglandin E_2; COX-1 mRNA; COX-2 mRNA; Nitric oxide synthase; eNOS; nNOS

1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder with exocrine glands as the site of intense immunological activity. Kerotoconjuntivitis sicca and xerostomia are the main symptoms but several extra-glandular manifestations may occur [1,2].

Lymphoproliferation and B cell activation are common and results in two important pathogenetic mechanisms: production of circulating auto antibodies and lymphocytic infiltratrion of the exocrine glands. Both of these processes could contribute to the wide range of clinical manifestations and to the immune-mediated destruction of the lachrymal and salivary glands, resulting in the loss of function which determines defining criteria of the syndrome [3].

The disease may extend from an autoimmune exocrynopathy to the manifestation of diverse extra-glandular symptoms. Neurological manifestations were

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doi:10.1016/j.intimp.2007.07.018

reported in patients with SS [4] and can include central nervous system involvement, cranial neuropathies, myelopathy and peripheral neuropathies [5]. Different types of cognitive dysfuctions have been described and most patients present memory disorders and impaired intellectual performance. Beside psychiatric disorders the affective disturbances, such as depression, anxiety, hypochondriasis and hysteria are the more commonly observed [6]. Neuropsychometric testing showed a high frequency of short-term memory defects and tomography scan analysis indicated abnormalities in the frontal lobes [7]. On neuropsychiatric testing, patients with primary SS (pSS) had significantly higher scoring rates for clinical anxiety and clinical depression compared with age and sex matched reference groups [8]. Immune-mediated hearing loss may occur in SS patients [9].

One scientific approach is based in the evidence that certain components of the serum IgG fraction from SS patients can recognize cerebral frontal cortex neuronal cells and these auto antibodies bind irreversibly and activate CNS muscarinic acetylcholine receptor (mAChR) [10]. Experimental evidence disclosed that these antibodies are directed at the agonist binding site of the molecule on the cell surface and behave as partial agonist, thus activating intracellular second-messenger signaling pathway coupled to mAChR; but interfering with subsequent ability of mAChR agonist to activate the same signaling pathways [11].

There is a well documented association between mAChR activity and the modulation of certain CNS functions such as memory, executive functions, attention and motor control [12]. Hence we proposed that patients with SS produce auto antibodies able to be fixed in cerebral mAChR and capable of causing CNS autonomic dysfunction.

Among the signaling events involved in mAChR activation in cerebral frontal cortex, the stimulation of nitric oxide synthase (NOS) activity [13] and the release of PGE_2 [14] were demonstrated. Prostaglandins (PGs) and nitric oxide (NO) represent some of the most relevant local mediators that participate in the modulation of many neuronal function, including learning and memory [15]. NO plays a key role in the pathophysiology of chronic inflammation and in neurodegenerative processes [16]. Moreover, and altered biosynthesis of prostanoids is clearly involved in neuroinflammatory processes [17].

The biosynthesis and release of NO and PGs share a number of similarities. Both are synthetized via identified bioenzymatic complex: cyclooxygenase (COX) for PGs and NOS for NO. These highly regulate biosynthetic pathways act on specific substrates (arachidonic acid and L-arginine, respectively) leading to pulsed release of nanomolar concentration of both mediators. Two major isoforms of NOS and COX enzymes have been identified. The constitutive isoforms (cNOS and COX-1) are found in virtually all normal conditions. On the other hand, in inflammatory setting, the inducible isoforms of these enzymes (iNOS and COX-2) are detected. However, in brain COX-2 is expressed constitutively and COX-1 is distributed in neurons throughout the brain, but it is most prevalent in forebrain, were PGs may be involved in complex integrative functions, such as modulation of the autonomic nervous system [18]. Human brain tissues contain equal amounts of mRNA for COX-1 and COX-2 [19].

Recent evidence has been accumulated indicating that there is a constant "cross talk" between NO and PGs biosynthesis pathways involving in the pathophysiological mechanisms underlying some relevant inflammatory disorders [15].

By considering that: 1. there is CNS alterations in pSS patients; 2. pSS is an autoimmune disorder associated with serum IgG auto antibodies targeting cerebral mAChR and with tissue inflammation; 3. both of these processes could contribute to the immune mediated CNS alteration associated with mAChR function and 4. the mAChR activation triggers simultaneously the biosynthesis of both proinflamatory local mediators NO and PGs. We considered it relevant to investigate whether serum anti frontal cerebral IgG antibodies from particular group of pSS patients by activation of mAChR are able to simultaneously trigger NO and PGE_2 production, emphasized the capacity of the auto antibody to induce early COX mRNA gene expression. Thus the possibility of a CNS-specific antigen antibody system in pSS patients displaying an agonistic-like activity could result in a deregulation of cerebral mAChR functional activity and in a immune-mediated cerebral inflammation.

2. Materials and methods

2.1. Subject and serologiocal test

The studied group comprised 15 women (aged 35–60 years) selected from the metropolitan area of Buenos Aires. The diagnosis of primary Sjögren Syndrome (pSS) was based on followed four of more criteria of Vitali et al. [20]. Patients complained of oral and ocular dryness and positive labial biopsies. Serologic tests were performed: anti-Ro/SS-A and anti-La/SS-B antibodies, rheumatoid factor (RF) and antinuclear antibodies (ANA), anti M_1 and anti M_3 mAChR peptide as describe in Table 1. Primary SS patients were selected from pSS patients that gave positive reaction for anti

Table 1
Serological test performed on different groups

Serological Test	Group I	Group II
ANA	11/15 (73.3%)	0/15 (0%)
RF	5/15 (33.3%)	0/15 (0%)
Anti-Ro/SS-A	7/15 (46.6%)	0/15 (0%)
Anti La/SS-B	5/15 (33.3%)	0/15 (0%)
Anti M_3 peptide	15/15 (100%)	0/15 (0%)
Anti M_1 peptide	15/15 (100%)	0/15 (0%)

cerebral frontal cortex membrane in indirect immunofluorescence and dot blot analysis [10]. All patients have a frontal lobe syndrome-related disorder according neuropsychological test alterations as previously reported [7]. Shortly, more frequency signs were: slowness, shifting capacity disorder, incapacity to resist cognitive conflict, programming capacity disorder and decrease verbal fluency. None of the patients received for at least one month of testing antidepressants or other medications. Control group consisted of 15 age-matched healthy subject. Patients and volunteers were informed of the purpose of the study and gave their consent according to the tenets of the Declaration of Helsinki.

2.2. Rat cerebral frontal cortex preparations

Male Wistar rats (obtained from the Pharmacology Unit, School of Dentistry, University of Buenos Aires) were housed in our colony in small groups and kept in automatically controlled lighting (lights on 08:00–19:00) and uniform temperature (25 °C) conditions. All animals were used at 3–4 months of age and were cared for in accordance with the principles and guidelines of the National Institutes of Health (NIH No. 8023, revised 1978). Efforts were made to minimize animal suffering such as: killing under anesthesia and reducing the number of animals, as well as using the same animal for all enzymatic assays.

2.3. Peptides

A 25-mer peptide E-R-T-M-L-A-G-Q-C-Y-I-Q-F-L-S-Q-P-I-I-T-F-G-T-A-M and 22-mer peptide Q-Y-F-V-G-K-R-T-V-P-P-G-E-C-F-I-Q-F-L-S-E-P corresponding to the amino acid sequence of the second extracellular loop of the human M_1 and M_3 mAChRs, respectively, were synthesized by F-moc-amino acids activated using HOBt/DCC (1-hydroxy benzo triazole/dicyclo hexyl carbodimide) strategy with an automatic peptide synthesizer Applied Biosystems Model 431A. The peptide was desalted, purified by HPLC, and subjected to amino-terminal sequence analysis by automatic Edman degradation with an Applied Biosystems 470 A Sequence.

2.4. Purification of Human IgG

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

2.5. ELISA procedure

Fifty microlitres (50 μl) of M_1 and M_3 mAChR peptides solution (20 μg/ml) or rat cerebral frontal cortex membranes (3–5 mg/ml protein) in 0.1 M Na_2CO_3 buffer pH 9.6 were used to coat microtiter plates (Costar) at 4 °C overnight. After blocking the wells, different dilutions of sera or purified IgG from pSS patients (Group I) and normal individuals (Group II) were allowed to react with the antigens for 2 h at 37 °C. The wells were then thoroughly washed with 0.05% Tween 20 in PBS. A 50 μl volume of goat anti-human IgG avidine–alkaline phosphatase (1:5000, v/v; Sigma) was added and incubated for 1 h at 37 °C. After several washing steps, *p*-nitrophenyl phosphate (1 mg/ml) was added as substrate and the reaction was stopped at 30 min. Optical density (O.D.) values were measured with an ELISA reader (Uniskan Laboratory System).

2.6. Determination of nitric oxide synthase (NOS) activity

NOS activity was measured in cerebral frontal cortex slices by production of [U–^{14}C]-citrulline from [U–^{14}C]- arginine according to the procedure previously described for brain slices [13]. Briefly, after 20 min in preincubation Krebs Ringer bicarbonate (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 incubated for 20 min under 5% CO_2 in O_2 at 37 °C. Tissues were then homogenized with an Ultraturrax in 1 ml of medium containing 20 mM HEPES pH 7.4, 0.5 mM EGTA, 0.5 mM 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.

2.7. PGE_2 assay

Rat cerebral frontal cortex slices (10 mg) were incubated for 60 min in 0.50 ml of Krebs Ringer bicarbonate (KRB) gassed with 5% CO_2 in oxygen at 37 °C. IgG was added 30 min before the end of incubation period and blockers 30 min before the addition of the IgG. Tissues were then homogenized into a 1.5 ml polypropylene microcentrifuge tube. Thereafter, all procedures employed were those indicated in the protocol of Prostaglandin E_2 Biotrak Enzyme Immuno Assay (ELISA) System (Amersham Biosciences, Piscataway, NJ, USA). The PGE_2 results were expressed as picogram/milligram of tissue wet weight (pg/mg tissue wet wt).

2.8. mRNA isolation and cDNA synthesis

Total RNA was extracted from rat cerebral frontal cortex slices by homogenization using guanidinium isothiocyanate method [13]. As previously described, a 20 μl reaction mixture contained 2 ng of mRNA, 20 units of RNase inhibitor, 1 mM dNTPs and 50 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). First-strand cDNA was synthesized at 37 °C for 60 min.

2.9. Quantitative PCR and Real Time PCR

Quantitation of COX-1 and COX-2 isoform mRNA levels was performed by a method that involves simultaneous co-amplication of both the target cDNA and a reference template (MIMIC) with a single set of primers. MIMIC for COX-1 and glyceraldehyde-3-phosphate dehydrogenase ($g_3$pdh) were constructed using a PCR MIMIC construction kit (Clontech Laboratories, Palo Alto, CA, USA). Each PCR MIMIC consists of a heterologous DNA fragment with 5′ and 3′-end sequences that was recognized by a pair of gene-specific primers. The sizes of PCR MIMIC were distinct from those of the native targets. The sequences of oligonucleotide primer pairs used for construction of MIMIC and amplification of cox isoform and g3pdh mRNAs are listed in Table 2. Aliquots were taken from pooled first-strand cDNA from the same group and constituted one sample for PCR. A series of 10-fold dilutions of known concentrations of the MIMIC were added to PCR amplification reactions containing the first-strand cDNA. PCR MIMIC amplification was performed in 100 μl of a solution containing 1.5 mM MgCl2, 0.4 mM primer, dNTPs, 2.5U Taq DNA polymerase and 0.056 mM Taq Start antibody (Clontech Laboratories). After initial denaturation at 94 °C for 2 min, the cycle condition was 30 s of denaturation at 94 °C, 35 s of extension at 58 °C and 35 s for enzymatic primer extension at 72 °C for 40 cycles for cox isoforms. The internal control was the mRNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase ($g_3$pdh). PCR amplification was performed with initial denaturation at 94 °C for 2 min followed by 30 cycles of amplification. Each cycle consisted of 35 s at 94 °C, 35 s at 58 °C and 45 s at 72 °C. Samples were incubated for an additional 8 min at 72 °C before completion. PCR products were subjected to electrophoresis on ethidium bromide-stained gels. Band intensity was quantitated by densitometry using NIH Image software. The cox mRNA levels were normalized with the levels of $g_3$pdh mRNA present in each sample, which served to check for variations in RNA purification and cDNA synthesis. The relative mRNA expression of cox was compared with those from the respective control group. For real time PCR amplification was performed using QuantiTect SYBR Green PCR and QuantiTect SYBR Green RT-PCR kits (in this case 250 ng or total RNA were used as starting sample) (Qiagen, Valencia, CA, USA) in an Cycler IQ Real-Time Detection System (Bio-Rat, Hercules, CA, USA) [21]. The protocol and the primers used were the same as that describe above. The primers were designed using Primer Premier 5 (Biosoft International, Palo Alto, CA, USA). Melting curves of all samples were always performed as a control of specificity. Analysis of relative gene expression was performed using the $2^{-\Delta\Delta C1}$ method and we compared COX isoforms to $g_3$pdh.

Table 2
Oligonucleotides of primers for PCR

Gene product	Sense	Antisense	Predicted size, bp
COX-1	5′ TAAGT ACCAG TGCTG GATGG 3′	5′ AGATC GTCGA GAAGA GCATCA 3′	160
COX-2	5′ TCCAA TCGCT GTACA AGCAG 3′	5′ TCCCC AAAGA TAGCA TCTGG 3′	242
$g_3$pdh	5′ ACCAC AGTCCA TGCCAT CAC 3′	5′ TCCAC CACCC TGTTG CTGTA 3′	452

Cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2) and glyceraldehyde-3-phosphate dehydrogenase ($g_3$pdh).

2.10. Drugs

Atropine, 4-DAMP, pirenzepine and N^Gmonomethyl-L-arginine (L-NMMA) were purchased from Sigma Chemical Company, Saint Louis, MO, USA; 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA), 1-[4,5-bis(4-methoxyphenyl)-2-thiazolyl]carbonyl-4-methylpiperazine hydrochloride (FR 122047), 5-bromo-2-(4-urophenyl)-3-[4-(methylsulfonyl)phenyl]-tiophene (DuP 697), L-NIO [N^5-1-iminoethyl)-L-ornithine] and NZ [N_2-propyl-L-arginine] were from Tocris Cookson Inc., Baldwin, MO, USA.

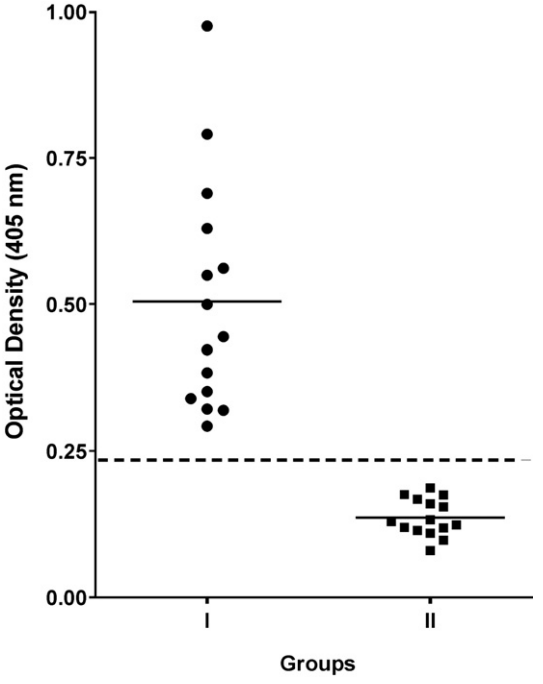

Fig. 1. Immune reactivity of anti cerebral frontal cortex membrane antibodies of sera from different groups: 15 pSS patients (Group I) and 15 normal subjects (Group II). Serum (1/30 dilution) was assayed on sensitized microplates with 50 μg/ml membranes. Dotted/dashed line, cut off values 0.24 (mean optical density (OD)±3 S.D. for Group II), solid lines, median OD values. *$p<0.001$ between Group I and Group II.

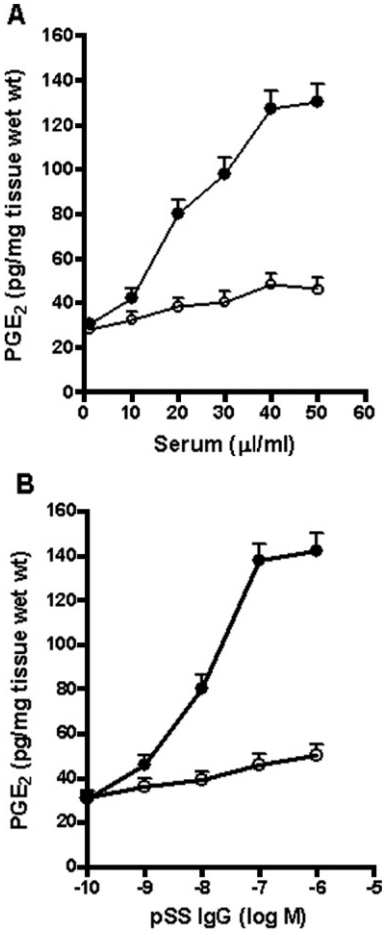

Fig. 2. PGE_2 production on rat isolated prefrontal cortex. PGE_2 production was measured after incubating cerebral frontal cortex slices with increasing concentration of serum (A) or IgG (B) from Group I (●) or Group II (○) during 30 min. Values represent the mean±S.E.M. of fifteen different patients. *$p<0.001$ between Group I and Group II sera or IgG.

2.11. Statistical analysis

Student's t-test for unpaired values was used to determine signicance levels. Analysis of variance (ANOVA) and post hoc test (Dunnett's Method and Student–Newman–Keuls test) were employed when a pairwise multiple comparison procedure was necessary. Differences between means were considered significant if $p<0.05$.

3. Results

3.1. Detection of serum antibody

Fig. 1 shows the immune reactivity of sera from different groups against cerebral frontal cortex membrane as coating antigens. One can see that the optical density (OD) values for sera from pSS patients (Group I) was 3 S.D. higher than those from normal control subjects (Group II).

3.2. Muscarinic cholinergic receptors mediated effect of serum auto antibodies on cerebral frontal cortex

As already shown the anti M_1 and anti M_3 peptide auto antibodies from pSS (Table 1) patients reacted with rat cerebral frontal cortex (Fig. 1). Knowing, that aminoacid sequence of rat and human M_1 and M_3 mAChR displays strong homology (93% and 89% respectively), we studied as M_1 and M_3 mAChR-mediated effect of auto antibodies from pSS on rat cerebral frontal cortex. The NOS activity and PGE_2 production were measured as representative of the local mediators that participates in the modulation of certain CNS functions.

Fig. 2A shows the ability of pSS sera to trigger PGE_2 release in a concentration-dependent manner, with 40 µg/ml proving the maximal response. The normal sera did not modify

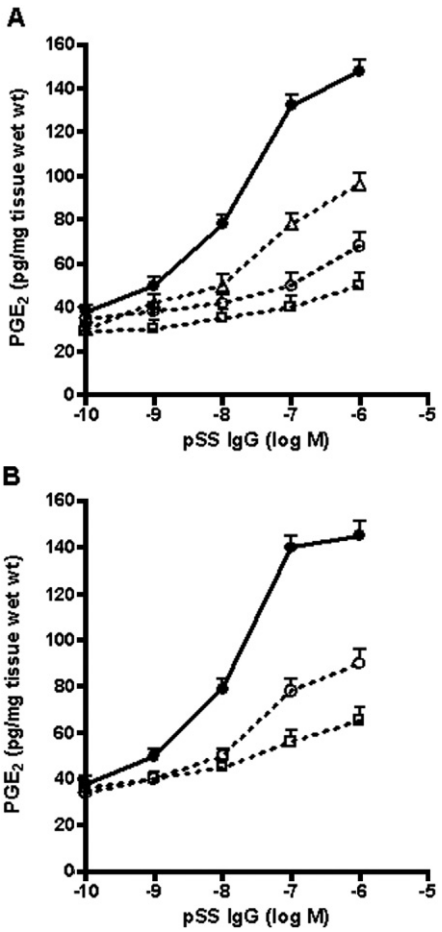

Fig. 3. Concentration-response curves of pSS IgG on PGE_2 production by rat isolated prefrontal cortex. A: The effects of pSS IgG alone (●) or in the presence of 1×10^{-6} M atropine (□), pirenzepine (○) and 4-DAMP (△) are shown. B: The effects of pSS IgG alone (●) or preincubated during 30 minutes with 1×10^{-5} M M_1 (○) or M_3 (□) synthetic peptide are also shown. Values represent the mean±S.E.M. of seven different IgG. *$p<0.001$ between pSS IgG alone and in presence of mAChR antagonists or peptides.

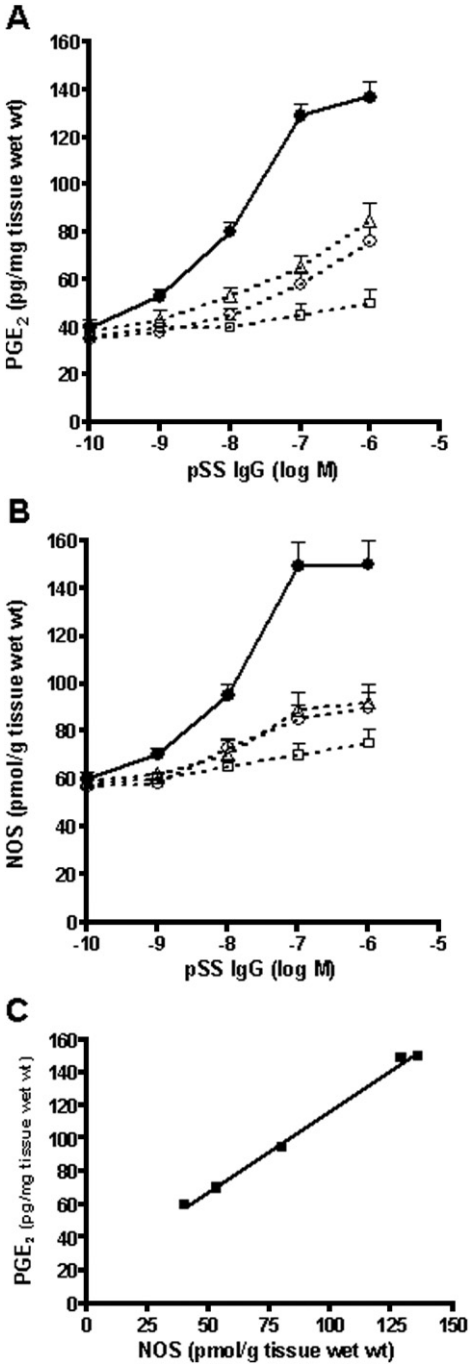

Fig. 4. A: Increase in PGE_2 production of pSS IgG alone (●) or in the presence of L-NMMA 1×10^{-4} M (□) or NZ 1×10^{-5} M (○) or L-NIO 1×10^{-5} M (△). B: Increase in NOS activity of rat prefrontal cortex by increasing concentrations of pSS IgG alone (●) or in the presence of L-NMMA 1×10^{-4} M (□) or NZ 1×10^{-5} M (○) or L-NIO 1×10^{-5} M (△). **C**: Correlation in the effect of pSS IgG on PGE_2 production and NOS activity (■). Results are mean±S.E.M. of seven pSS IgG performed by duplicate. *$p<0.001$ between pSS IgG alone and in presence of different inhibitors.

the release of PGE_2. Fig. 2B shows that IgG from the same pSS patients triggered PGE_2 release in a concentration-dependent manner, being 1×10^{-7} M pSS IgG the maximal response and normal IgG did not have effect.

As can be seen in Fig. 3A pSS IgG triggered PGE_2 generation was inhibited in the presence of mAChR selective antagonist like pirenzepine (M_1) and 4-DAMP (M_3) and the muscarinic antagonist, atropine. When pSS IgG was pre-incubated with M_1 and M_3 synthetic peptides corresponding to the second extracellular loop of human M_1 and M_3 mAChR, the generation of PGE_2 was inhibited (Fig. 3B).

To determine if the endogenous NO participate in pSS IgG-induced PGE_2 generation, cerebral frontal cortex were incubated with particular NOS isoform inhibitors. Fig. 4A shows the inhibition of NOS by 1×10^{-4} M L-NMMA or by the specific inhibitors of endothelial NOS (eNOS), 1×10^{-5} M L-NIO and neuronal NOS (nNOS) 1×10^{-5} M NZ decreased the pSS IgG-generated PGE_2. As control, 5×10^{-4} M L-arginine reversed the L-NMMA effect (data non shown). Additional studies were performed in order to assess if pSS IgG is able to stimulated NOS activity. Fig. 4B shows that auto antibodies increased NOS activity in a concentration-dependent manner. Moreover, 1×10^{-5} M L-NIO and 1×10^{-5} M NZ decreased the pSS IgG-stimulated NOS activity. Fig. 4C demonstrated that under identical experimental conditions a significant correlation ($\alpha=0.05$) between pSS IgG stimulated PGE_2 production and NOS activity was founded (Pearson r: 0.9981; $p=0.0001$).

In order to discern, which arachidonic acid (AA) cascade enzyme are implicated in the pSS IgG-generated PGE_2, several inhibitors of this cascade reaction were used. It can be seen in Fig. 5 that inhibition of PLA_2 by OBAA (5×10^{-6} M) or COX-1 by FR122047 (5×10^{-8} M) prevented the stimulatory action of pSS IgG induced PGE_2 production, while COX-2 inhibition

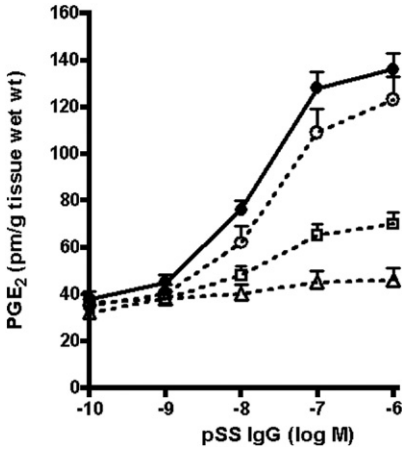

Fig. 5. Concentration-response curves of pSS IgG on PGE_2 production by rat prefrontal cortex in the absence (●) or in the presence of 5×10^{-6} M OBAA (△) or FR122047 5×10^{-8} M (□) or DuP 696 5×10^{-8} M (○). Results are mean±S.E.M. of seven pSS IgG performed by duplicate. *$p<0.001$ between pSS IgG alone and in presence of FR122047 or OBAA.

by DuP 697 (5×10^{-8} M) was without effect. Higher concentrations (5×10^{-7} M and 5×10^{-6} M) of DuP had no effect in our system (data not shown). Using 5×10^{-6} M of $AACOCF_3$, a drug know to selectively inhibited PLA_2, the effect of the antibody was inhibited about 98%±8 ($n=5$) as OBAA did.

With the use of specific oligonucleotide primers for COX-1 and COX-2 mRNA gene expression, RT-PCR amplified products showed single clear bands of the predicted size (Fig. 6). Semi-quantitative reversed transcription polymerase chain-reaction analysis demonstrated that stimulation with pSS IgG (1×10^{-7} M) triggered increase in COX-1 mRNA levels but did not modify COX-2 mRNA levels (Fig. 6A). A reduction in PGE_2-induced elevation of COX-1 mRNA levels was observed in the presence of atropine, L-NMMA, OBAA and mAChR peptide M_1 and M_3 (Fig. 6B). Similar results were obtained with the use of Real Time PCR (Table 3). The relative

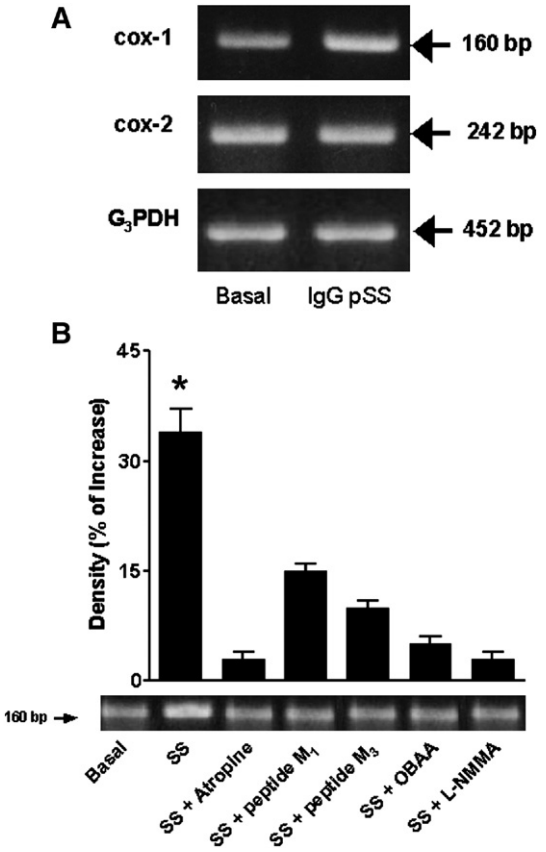

Fig. 6. A: RT-PCR products obtained from pSS IgG effect on COX-1 and COX-2 mRNA expression. B: semiquantitative RT-PCR analysis for pSS IgG induced increase in COX-1 mRNA levels in cerebral frontal cortex preparations. Preparations were incubated for 1 h with 1×10^{-7} M pSS IgG alone or in the presence of 1×10^{-7} M atropine, 1×10^{-5} M peptide M_1, 1×10^{-5} M peptide M_3, 5×10^{-6} M OBAA and 1×10^{-4} M L-NMMA. Basal values: Basal. RT-PCR products obtained from the analysis are shown. pSS IgG differ significantly from basal values with $p<0.0001$.

Table 3
Relative COX-1 mRNA expression by Real Time PCR

Addition	Relative values
Basal	0.61±0.07
pSS	1.15±0.11*
pSS+Atropine (1×10^{-7} M)	0.70±0.07
pSS+M_1 peptide (1×10^{-5} M)	0.91±0.08
pSS+M_3 peptide (1×10^{-5} M)	0.79±0.08
pSS+OBAA (5×10^{-6} M)	0.73±0.07
pSS+L-NMMA (1×10^{-4} M)	0.69±0.07

Relative COX-1 mRNA levels mean values±SEM of three determinations. *$p<0.01$ pSS versus basal.

COX-2 mRNA expression was: basal 1.12±0.1 and in the presence of pSS IgG 1.18±0.2 with three simultaneous experiments. Results point to the simultaneous participation of mAChR M_1 and M_3 subtypes, NO and PLA_2 in the ability of pSS IgG up-regulated COX-1 expression.

4. Discussion

Immune-mediated neurological manifestation in SS have been described [9] but the precise immune mechanism remain unclear. Here, we demonstrated the possible role of auto antibodies against cerebral mAChR on central nervous system (CNS) manifestations in pSS patients.

It is interesting that most common CNS manifestations of pSS patients were alterations in cognitive function with subtle abnormalities in the frontal lobes [7,22] and many of the cognitive aspects of the disease are linked to cholinergic dysfunction of brain tissue [23,24]. Therefore, the nature of the cholinergic CNS dysfunction in pSS patients, could be related with the effect of the anti brain auto antibodies on the muscarinic cholinergic system.

In this sense, using synthetic peptides with identical aminoacid sequence of the second extracellular loop of human M_1 and M_3 mAChR; we established that both, M_1 and M_3 mAChR subtypes, are target for the anti brain auto antibodies described in pSS patients. These data confirm previous results [10]. Knowing that the aminoacid sequence of rat and human M_1 and M_3 cholinergic synthetic peptides have strong homology (92%–86%), we study the mAChR-mediated effect of auto antibodies from pSS patients on rat cerebral frontal cortex.

The anti brain auto antibodies were not only able to interact molecularly with the human M_1 and M_3 mAChR peptide but also displayed agonistic activity which triggered PGE_2 production and NOS activity. Therefore, these anti neural receptor antibodies could be

involved in the ability of target organs to carry out their biological function. Studies on signaling events involved in mAChR activation of rat cerebral frontal cortex showed that stimulation of NOS and PGE_2 were implicated [13,14,25].

In this study we show that activation of M_1 and M_3 mAChR of rat cerebral frontal cortex preparations by anti brain pSS IgG triggers increased generation of PGE_2. This is preceded by NOS activation, which in turn catalyzes PLA_2-AA release and induces immediate early COX-1 mRNA gene without affecting COX-2 mRNA levels. Our data indicate that the endogenous NO signaling system is a key factor for pSS IgG induced PGE_2 generation and is the putative transcriptor factor able to increase the rate of transcription of COX-1 in response to mAChR activation. The nNOS and eNOS, are activated by anti brain pSS IgG and both isoforms participate on anti brain pSS IgG-induced PGE_2 generation. Previously we reported that pSS IgG activated M_3 mAChR increase eNOS and nNOS activity and mRNA gene expression, while the pSS IgG activated M_1 mAChR increase only nNOS activity and nNOS mRNA levels [10]. The positive PGs regulation by pSS IgG observed in this paper was mostly due to PLA_2 and COX-1 as it was prevented by specific blockade of these two enzymes.

The fact date that FR122047, known to selectively inhibit COX-1, has proven to be effective in preventing pSS IgG-stimulated PGE_2 generation, suggests that this pSS IgG action is under control of COX-1 mRNA, in agreement with the positive regulation of COX-1 mRNA without changes in COX-2 mRNA expression. The lack of COX-2 specific inhibitor confirmed this statement. COX-1 is prevalent in forebrain were PGs may be involved in complex integrative function, such as modulation of the autonomic nervous system [18]. Moreover, COX-1 activity rather than COX-2 has been related with neuronal excitotoxicity and cortical neuronal death [26]. Such functional dichotomy helps to explain the preferential COX-1 mRNA pSS IgG effect. On the basis of our results we suggest that anti brain pSS IgG-activated mAChR triggered NO accumulation that in turn co-stimulates both PGE_2 production and the induction of COX-1 mRNA gene expression through the activation of NOS activity.

An altered biosynthesis of prostanoids is clearly involved in neuroinflammatory processes [17]. Indeed, COX-1 positive microglia accumulated significantly at perilesional area and in the developing necrotic core after injury, being the number of COX-1 positive cells persistently elevated up to four weeks following injury [27]. On the other hand, over expression of COX-1 and subsequent abnormal release of prostanoids, has been described after exposure of brain cells to different inflammatory agents, such as endotoxin, cytokines and *Escherichia coli* LPS [28].

The mechanism by which cholinergic auto antibodies stimulates PGE_2 and COX-1 mRNA levels may be explained by efficient coupling of Gq-protein to M_1 and M_3 frontal cerebral mAChR [14]. Thus, it was demonstrated that the activation of M_1 and M_3 mAChR increased intracellular calcium concentrations via phospholipase C, which in turn triggers a cascade reaction involving calcium/calmodulin leading to activation of NOS increasing NO production that up-regulated subsequently COX-1 mRNA levels and PGE_2 generation.

Thus, on the basis of our results we postulated that early agonistic-promoting activation of mAChR initiated by antibodies, bind to and persistently activate cholinoceptor resulting in the production of large amount of proinflammatory and cytotoxic NO and PGs. The release of NO and PGs by the induction of nos-mRNA and cox-mRNA genes expression could be contribute to immune neuroinflammation. Later on, the agonistic activity displayed by these auto antibodies could induce desensitization, internalization and/or intracellular degradation of the mAChR, leading to a progressive decrease of cerebral M_1 and M_3 mAChR expression and activity, resulting in higher cognitive function alteration including synaptic plasticity and memory present in pSS patients.

Acknowledgement

We thank Mrs. Elvita Vannucchi and Fabiana Solari for their outstanding technical assistance. This research has been supported by grants UBACYT (OD 14) from Buenos Aires University and PIP (2532) from CONICET.

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